EFFECTS OF EARLY ONTOGENETIC SENSITIVE PERIODS ON THE GENERALIZATION OF RISK AND SAFETY INFORMATION IN WOOD FROG TADPOLES (*LITHOBATES SYLVATICUS*)

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STEPHEN PECYLAK

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Head of the Department of Biology

Collaborative Science Research Building

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University of Saskatchewan

Saskatoon, SK

Canada, S7N 5E2

Dean of the College of Graduate and Postdoctoral Studies

116 Thorvaldson Building

110 Science Place

University of Saskatchewan

Saskatoon, SK

Canada, S7N 5C9

Abstract

Environments often change within an organism's lifetime. The ability to react and adapt to these changes is referred to as plasticity. Periods of development with heightened plasticity are called sensitive periods. Events experienced during sensitive periods can have disproportionate effects later in life across multiple phenotypes, a phenomenon called phenotypic resonance. Originally based on the phenomenon of phenotypic resonance, the phenomenon of cognitive resonance is described as the disproportionate effect a sensitive period has on the way information is used by an individual. Cognitive resonance has been studied using risk and safety information, but not on other cognitive processes such as generalization. This thesis focused on the effects of sensitive periods on the generalization of safety and risk related information. Wood frog tadpoles (*Lithobates sylvaticus*) were chosen as the model system and embryonic development as the sensitive period. In the first experiment, tadpoles were trained to recognize brook trout as a predator using a pairing with conspecific alarm cues, which are innately recognized as indicating risk. Tadpoles were then exposed to one of the following test odours to form a generalization gradient based on phylogenetic relatedness: brook trout, splake, tiger trout, rainbow trout, or goldfish. Tadpoles trained as embryos that brook trout was risky partially generalized risk to splake, tiger trout, and rainbow trout, which are all members of Salmonidae, but not to the distantly related goldfish. Tadpoles trained that brook trout was risky as larvae only generalized risk to splake and tiger trout, both of which are hybrids of brook trout. The second experiment followed similar procedures to the first. However, tadpoles were trained to recognize brook trout odour as safe through a process of repeated unpaired exposures called latent inhibition. Each tadpole was then taught one of the aforementioned test odours as risky through one paring with alarm cues. Tadpoles trained as embryos that brook trout was safe generalized safety partially to splake, tiger trout, and rainbow trout, but not to goldfish. Tadpoles trained that brook trout was safe as larvae only generalized to splake, the intra-genus hybrid with brook trout. These two studies indicate that embryonically exposed tadpoles generalize to more species than do larval tadpoles for both safety and risk related information. My research is among the first studies to delve into the effects of cognitive resonance and could help to further understand the effects of early development on cognitive abilities. This thesis also has implications for fields where knowledge of early development might make a difference, such as behavioural conservation and human cognitive development.

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Chapter 1: Introduction

1.1: Plasticity and Sensitive Periods:

1.1.1: Plasticity:

Population level variation in a trait, an emergent property of inter-individual differences, serves as one of the foundational pillars of natural selection (Darwin, 1859 as assessed by Cauchoix & Chaine, 2016), as some individuals are more likely to survive than others as the environment changes. An example of population level variation is the three throat colour variants in male side-blotched lizards *Uta stansburiana*, which correspond to either a harem mating strategy, a mate guarding strategy, or a sneaker male strategy (Sinervo & Lively, 1996). The relative fitness of each variant depends on the frequency of the other two variants within the population, which itself fluctuates as one variant gains an advantage across generations. The suite of genetic factors is called a genotype (Johannsen, 1911) and is usually studied with regards to the genes of interest that affect a phenotype. Many environments fluctuate too rapidly and too stochastically for a strict genotype-phenotype matching to be adaptive (see Nylin & Gotthard, 1998; O'Connor *et al.,* 2014; Fawcett & Frankenhuis, 2015 for examples) and population level variation takes at least one generation to respond to environmental change (as seen with side-blotched lizards). In a rapidly fluctuating environment, having a single genotypically-determined phenotype means that an individual adapted to one environment may end up mismatched to either the current environment or a potential future environment.

The way around the problem of fixed phenotype is to have the individual itself also display variability in the phenotypes that they can express. The variation can be genetically encoded, such as in the classic example of solitary and gregarious forms in locust (see Ernst *et al.,* 2015 for review). An individual locust can change between a solitary and gregarious morph, both of which are genetically encoded but only one is expressed at a time, based on factors like population density, reduction in food availability, and stress. However, individuals can only have one suite of genetic information to draw from, so phenotypic variation that is strictly genetically determined will be limited by the genetic information that is already present. One method for altering phenotypes to match a rapidly changing environment without genetically encoding a limited number of morphs is through plasticity. Plasticity is the well-studied phenomenon described as either "the capacity for reactive change" (Lövdén *et al.,* 2010) or "the potential for an organism to produce a range of different, relatively fit phenotypes" (DeWitt *et*

al., 1998). Phenotypic plasticity is a type of plasticity defined as the generation of multiple phenotypes from one genotype (Nylin & Gotthard, 1998) and deals more directly with how much the phenotype can change. One example of phenotypic plasticity is the classic example in Lüning (1992), where *Daphnia* showed alterations in neck spine presence and tail spine length as a result of the presence of the predator *Chaoborus*.

Behavioural plasticity is the ability of an organism to change its behavioural responses as a result of its environment (Mery & Burns, 2010). A related phenomenon is cognitive plasticity, which can be defined as either the ability for the brain to alter during development, the process of learning, the degree to which performance is altered as a result of experience with a cognitive task, or the capacity to reactively alter the boundary conditions of flexibility for a labile trait (all reviewed in Lövdén *et al.,* 2010). It is this last definition that will be used in this thesis when referencing the plasticity of cognitive responses. Plasticity of flexible or labile traits are expected to evolve under situations where the environment changes rapidly, cues in the environment give a consistent amount of information about the environment, the cost/benefit trade-off for plasticity is low, and the trait can be inherited (Mery & Burns, 2010).

1.1.2: Sensitive Periods and Embryonic Learning:

While there are clear benefits to plasticity, there are also potential costs and limits (DeWitt *et al.,* 1998). For example, it is possible that plastic phenotypes have more difficulty reversing or that plastic phenotypes have trouble integrating multiple selective forces (Auld *et al.,* 2009). The classic example is Relyea (2003), where gray tree frog (*Hyla versicolor*) tadpole morphology could track predator presence in order to develop deeper bodies when presented a dragonfly predator early in life, but could not generate as extreme a phenotype when presented the same predator later in life. The ease of altering phenotypes early in life but not later in life indicates a sensitive period, which is defined as either the opening of plasticity during a period of ontogenetic development (Fawcett & Frankenhuis, 2015; Fuhrmann *et al.,* 2015) or as a period of ontogenetic development in which the environment disproportionately affects an individual's phenotype (Fawcett & Frankenhuis, 2015). Developmental effects, such as sensitive periods, are one of the cornerstones of modern ethology (Tinbergen, 1963) and sensitive periods specifically follow from many theoretical models of behavioural development (Fawcett & Frankenhuis, 2015; Stamps & Frankenhuis, 2016; Stamps & Krishnan, 2017).

Critical periods are a notable type of sensitive period that have a defined start, a defined end, a specifically affected system or systems, involve specific stimuli (Colombo, 1982), and the alteration becomes nearly irreversible (Panchanathan & Frankenhuis, 2016). Critical periods are often also seen as being crucial for further development of the involved system (Colombo, 1982 citing Fox, 1970; Fox *et al.,* 2010). Critical periods were first popularized by the phenomenon of imprinting in birds (as popularized by Lorenz, 1937), whereby a fledgling bird will fix onto an individual as a parental figure within the first 13-16 hours after hatching (Ramsay & Hess, 1954).

Both the aforementioned changes occurred during early development, which is an expected sensitive period. Early development is the first time an individual can sample cues from the environment (Stamps & Frankenhuis, 2016) and there is an expectation that uncertainty will decrease as an organism's absolute age increases, resulting in the fixation of a phenotype over time (Fawcett & Frankenhuis, 2015). In aquatic systems, information about the environment can be obtained while still in the egg (Hepper & Waldman, 1992; Nelson *et al.,* 2013) usually using olfactory information. Studies on fish embryos have also shown that brain size is affected by environmental conditions experienced in the egg (see Jonsson & Jonsson, 2014 for review). It is thought that as the brain develops, simple cognitive abilities develop before more complex cognitive abilities (Fox *et al.,* 2010), as the complex cognitive abilities require the simple cognitive ability neural circuits to be fixed before they can develop, although complex cognitive neural circuits can also mask simple cognitive abilities. The environmental circumstances that influence the development of the simple cognitive abilities could become more fixed as the more complex cognitive abilities develop.

1.1.3: Carry-Over Effects and Phenotypic Resonance:

Sensitive periods can result in carry-over effects, where the results from one event influence the results of other events (Harrison *et al.,* 2011; O'Connor *et al.,* 2014). Carry-over effects were first defined as the effect that a patient's initial treatment would subsequently have on other treatments (see O'Connor *et al.,* 2014 for review), but was co-opted by the ecology literature to mean when the success of one breeding season influences the success of future breeding seasons (see Harrison *et al.,* 2011 for review) and has since been applied to a variety of other behavioural, life history, breeding, as well as seasonal transition states such as migration and hibernation (O'Connor *et al.,* 2014; Taborsky, 2017). One well studied carry-over effect is

the silver spoon effect (Grafen, 1988; Monaghan, 2007; Taborsky, 2017 ch. 1.2.), which occurs when individuals born with greater initial access to resources have a long-term fitness advantage over those that had lower initial access to resources, although there is some debate as to whether such early developmental effects constitute carry-over effects (Harrison *et al.,* 2011), which would depend on the underlying mechanisms for carry-over effects compared to the underlying mechanisms of early developmental effects (O'Connor *et al.,* 2014).

A relatively recently described type of carry-over effect is phenotypic resonance, which is defined as the disproportionate influence a single event or series of events during a sensitive period may have on later phenotypic development (Massot & Aragón, 2013). Massot & Aragón (2013) took advantage of a phenomenon similar to the silver-spoon effect by feeding *Zootoca vivipara* lizards a single meal during the first two days post-hatch while not feeding others. This single event resulted in the unfed lizards showing altered dispersal, higher recapture rates, lower survivorship after accounting for recapture rate in the model, larger clutches, less variable offspring sex ratios, and in some cases slower growth rates. A later study (Whiteside *et al*., 2016) using pheasants (*Phasianus colchicus*) found a similar phenomenon where exposure to a spatially complex environment during early development had a within season effect on survival, aggressive conspecific interactions, male morphology, and some effects on spatial memory. Phenotypic resonance is a type of long-term and unconditional carry-over effect, unconditional meaning not dependent on or modulated by later events (see Taborsky, 2017 ch. 1.2. for definitions), that focuses on a narrower range of initial events and a broader range of effected traits than other carry-over effects.

1.1.4: Cognitive Resonance:

Cognitive resonance is defined as the alteration in the interpretation of information caused by a cognitive event happening during one ontogenetic stage as compared to the same event occurring at a different ontogenetic stage (Ferrari *et al.,* 2019) and is based on phenotypic resonance. Ferrari *et al.* (2019) trained wood frog (*Lithobates sylvaticus*) tadpoles to recognize a potential predator as being either risky or safe during embryonic development. Embryonically trained tadpoles retained predation information for longer than larval trained tadpoles that were trained to recognize the same predator. Embryonically trained tadpoles taught to recognize an odour as safe took more training sessions to reverse the previous association than larval trained tadpoles that were trained to recognize the same odour as safe. The alteration in the retention of

behavioural responses indicates that an associative learning event can influence an aspect of memory depending on when the information was initially presented, although the information presented is not considered critical development as in imprinting. Cognitive resonance differs from other non-critical early developmental cognitive phenomena, such as consolidation of early life information and priming of responses due to early physiological states (Taborsky, 2017 ch. 1.2.), in that cognitive resonance looks at the plasticity of responses and potentially affects multiple cognitive processes.

1.2: Cognitive Ecology:

1.2.1: General Cognitive Ecology Framework:

Cognitive ecology is the study of cognitive abilities under ecological and evolutionary constraints (Real, 1993; Dukas, 1998). The framework for the subfield treats cognition as an evolved trait that is adapted towards ecologically relevant problems, so cognition studies should aim for ecologically relevant situations or stimuli (Real, 1993; Cauchoix & Chaine, 2016). For example, naturally occurring populations of the chickadee *Poecile gambeli* from higher elevations show faster caching rates, longer retention of food association, and faster associative learning (Freas *et al.,* 2012; Kozlovsky *et al.,* 2015), hypothesized to be due to an adaptive need for chickadees in harsh environments to have better problem solving abilities or due to temperature effects on brain morphology. There is interest about the limits and constraints of cognitive and behavioural traits in cognitive ecology rather than just the adaptive benefits (Sih & Giudice, 2016). Phenotypic and cognitive resonance could both be considered cognitive ecological constraints, as both have been shown to constrain cognition while using ecologically relevant stimuli/events to initiate the resonance-type phenomenon (Whiteside *et al*., 2016 for phenotypic resonance, Ferrari *et al.,* 2019 for cognitive resonance).

Resonance-type phenomena may serve as constraints by affecting the ecological rationality of a given response. Ecological rationality is the degree to which a ruleset matches the context and problem to be solved (Hutchinson & Gigerenzer, 2005; Volz & Gigerenzer, 2012). The rulesets available depend on the adaptive toolbox at the individual's exposure, which contains three major factors: the type of information obtained, the environmental restrictions in processing that information, and the suite of rules the individual possesses for processing the information (Gigerenzer & Selten, 2002). The timing of events likely plays a role in determining which rulesets are used, as cognitive resonance leads to two separate memory retentions in spite

of the identical information presented to both groups (Ferrari *et al.,* 2019). This indicates that at least one group had fixed on a different ecologically rational outcome despite the same cues being used.

1.2.2: Wood Frogs as a Model System:

There are several reasons for using anuran tadpoles for studying cognitive ecology, specifically cognitive resonance. Anurans undergo metamorphosis, which leads to an ecological niche shift. It is known that *Rana ridibunda* can maintain exploratory/activity behavioural syndrome axis across the metamorphic boundary (Wilson & Krause, 2012) and predation/competition risk can affect brain region size post-metamorphosis on *Rana temporaria* (Trokovic *et al.,* 2011). Studying tadpoles can provide information on fitness variation in adult frogs. Tadpoles can also undergo embryonic learning (Hepper & Waldman, 1992), which has largely focused on similar types of olfactory stimuli that were used to demonstrate cognitive resonance (Hepper & Waldman, 1992; Ferrari & Chivers, 2008; Ferrari & Chivers, 2009a; Ferrari & Chivers, 2010; Garcia *et al.,* 2017; Supekar & Gramapurohit, 2017).

The Skipper Frog *Euphlyctis cyanophlycti* might be able to learn olfactory cues about their environment before the neural fold has formed (Supekar & Gramapurohit, 2017), the mechanism behind which is unknown. The stage when neural fold formation occurs can be identified due to the well-known staging index for Anuran embryonic and larval development (Gosner, 1960). Olfactory system development occurs just after neural tube closure in he African Clawed Frog *Xenopus laevis* (Graziadei & Monti-Graziadei, 1992), matching what was seen in the Skipper Frog (Supekar & Gramapurohit, 2017) although a different staging index is used in the African Clawed Frog (Nieuwkoop, 1956; as assessed by Graziadei & Monti-Graziadei, 1992). Wood frog embryonic learning pre-exposures occur around this stage as well (Ferrari & Chivers, 2009a; Ferrari & Chivers, 2009b), so it appears likely that wood frog development follows a similar trajectory.

Wood frog life history characteristics make them of interest over other anurans. Wood frogs primarily breed in ephemeral pools where the suite of present organisms is both less consistent and more transient than in permanent bodies of water (Colburn *et al.,* 2008). As such, there is a greater impetus for wood frog tadpoles to start with minimal assumptions about the environment and to rapidly learn the local predator complement, as presented by the predator recognition continuum hypothesis (Ferrari *et al.,* 2007a; Ferrari & Chivers, 2009a).

Accordingly, wood frogs do not recognize trout, goldfish, salamanders, newts, or frogs as innately dangerous but can be taught such (Ferrari & Chivers, 2009a; Ferrari *et al.,* 2009; Chivers & Ferrari, 2013; Chivers *et al.,* 2015; Ferrari *et al.,* 2016). Wood frogs also overwinter as adults (Storey & Storey, 1984), which could lead to another sensitive period after the thaw.

Wood frog tadpoles are a convenient model system as the antipredator behaviour has been standardized into a single behavioural metric using line crosses (Ferrari *et al.,* 2007b). Wood frogs are found throughout most of the North American continent and are considered valuable for monitoring the conservation of ephemeral pools (Baldwin *et al.,* 2006), as well as being one of the most common prey species (see Colburn *et al.,* 2008, ch. 6 for review on ephemeral pool invertebrates). As such, they are easy to obtain and different populations from geographically separated regions can easily be compared. Finally, wood frogs lay large number of eggs within a given clutch (Skidds *et al.,* 2007), which allows for ample replication.

1.3: Predation Risk and Classical Conditioning:

1.3.1: Risk Aversion and Risk Association:

Risk aversion, uncertainty aversion (see Volz & Gigerenzer, 2012 for review on risk and uncertainty aversion), and loss aversion (Mathis & Steffen, 2015) have been well studied in psychology and serve as a major part of behavioral economics. Risk aversion refers to perfect information systems, whereas uncertainty aversion refers to situations when uncertainty is perceived as costly. These two contexts use different decision-making processes, as stated by Stiglitz (2010): "It simply wasn't true that a world with almost perfect information was very similar to one in which there was perfect information" (p. 243, as retrieved by Volz & Gigerenzer, 2012). In order to reduce the uncertainty associated with a source of potential risk, animals can use associative learning (see Shettleworth, 2001), which is the ability to draw a connection between two stimuli: one that causes a response (the unconditioned stimulus or US) and one that does not (the conditioned stimulus or CS), following from classical conditioning (see Pavlov & Gantt, 1928, for translations of Pavlov's lectures). The two stimuli become associated such that one predicts the other, allowing both to elicit a response. Ecologically relevant studies into risk association usually use predation threats (see Shettleworth, 2001, pg. 281-282).

1.3.2: Alarm Cues:

Risk association is often done through the use of alarm cues, originally called *schreckstoff* (Frisch, 1942; Rehnberg & Schreck, 1987), in aquatic systems. Alarm cues are a chemical cocktail released after the epidermis (see Chivers & Smith, 1998; Chivers *et al.,* 2007) is pierced and are innately recognized. Due to the circumstances of production, alarm cues are considered a highly informative indicator of potential risk (Chivers *et al.,* 2012), to the point where a single presentation can elicit a response days after the initial presentation (Gonzalo *et al.,* 2010; Ferrari *et al.,* 2012). The exact composition of alarm cues is not known, although some studies have begun to determine components of alarm cues for some fish (Brown *et al.,* 2003) and amphibians (Fraker *et al.,* 2009). Alarm cues serve as a useful stimulus-based mechanism for learning about risk without having to encounter the source of risk directly, allowing for faster responses to predators. This can increase survival, as demonstrated by western spadefoot toad tadpoles (*Pelobates cultripes*), which can survive longer against a crayfish predator (*Procambarus clarkia*) when first taught that the crayfish is a predator using alarm cues (Polo-Cavia & Gomez-Mestre, 2014). This increase in survival translates to field experiments as well, as demonstrated by rainbow trout (Mirza & Chivers, 2000). Alarm cues were also one of the original contexts used to study cognitive resonance (Ferrari *et al.,* 2019).

1.4: Latent Inhibition:

A less studied phenomenon related to risk association is learning about safety or inhibitory stimuli. One method for safety learning follows from the classical conditioning phenomenon of latent inhibition (Ferrari & Chivers, 2011), which makes it harder to form any association with a trained or excitatory stimulus through repeated pre-exposure to the inhibited stimulus. This indicates passive safety, instead of either a neutral stimulus or conditioned safety through conditioned inhibition (Ferrari & Chivers, 2011). In order to determine if latent inhibition has occurred, the safe stimulus is presented with a recognized US, such as alarm cues to see how long it takes for an association to form (Ferrari & Chivers, 2008; Ferrari & Chivers, 2011) in a process known as reversal learning. If reversal learning was successful, presentation of the CS alone should result in a conditioned response (CR), which is the same as the unconditioned response (UR) from the negative stimulus. Otherwise, the cue is said to be latently inhibited.

Latent inhibition in animal systems was first described in depth using goats and sheep with visual CS and electricity as the US (Lubow & Moore, 1959), and continues to be studied in animals for comparisons to human anxiety-like behaviours (Tsakanikos & Reed, 2019). Cognitive ecology studies in aquatic systems frequently use olfactory cues as the inhibited CS with alarm cues as the reversal learning US, such as in damselfish *Pomacentrus moluccensis* (Mitchell *et al.,* 2011), amphibians (Ferrari & Chivers, 2008; Ferrari & Chivers, 2011; Gonzalo *et al.,* 2013), and minnows *Pimephales promelas* (Ferrari & Chivers, 2006). Mitchell *et al.* (2011) showed that it takes six reversal learning sessions using AC in damselfish to reverse latent inhibition formed by six pre-exposures. Ferrari & Chivers (2008) showed that even embryonically trained wood frogs can learn inhibitory as well as excitatory stimuli. However, ecologically relevant latent inhibition studies are noticeably lacking in comparison to risk association paradigms. Safety through latent inhibition was also one of the contexts used in the initial study of cognitive resonance (Ferrari *et al.,* 2019).

1.5: Generalization:

A separate cognitive process that could be equally affected by cognitive resonance and applied to both risk and safety related information is stimulus generalization. Stimulus generalization is the process whereby the response to a trained CS appears for a similar untrained stimulus based on the degree of similarity (Mednick & Freedman, 1960; Ghirlanda & Enquist, 2003). When multiple novel stimuli are subsequently tested, with varying degrees of relation to the initially trained stimulus, a generalization gradient can be constructed (Kalish & Guttman, 1957). Antipredator generalization was first demonstrated in Griffin *et al.* (2001) by showing that wallabies taught the silhouette of a fox was risky generalized their antipredator responses to the silhouette of a cat but not a goat. In aquatic systems, antipredator generalization can be studied through the use of alarm cues (Ferrari *et al.,* 2007a; Ferrari *et al.,* 2009; Ferrari & Chivers, 2009a; Ferrari & Chivers, 2010; Chivers *et al*., 2016; Ferrari *et al.,* 2016). The generalization gradient is normally formed using taxonomic relationships, which could provide a fitness advantage if it is assumed that phylogenetically related individuals occupy similar ecological niches (Ferrari *et al.,* 2007a; Losos, 2008). Fish can also generalize from a presented cocktail of odours to the individual components (Darwish *et al.,* 2005), which may be ecologically relevant if hybrid species odours are a cocktail of the parental odours (Chivers *et al.,*

2015). Safety generalization is a less studied phenomenon, but has been studied in wood frogs (Ferrari & Chivers, 2011).

Risk association through alarm cues and generalization both allow for learning about risk without having to experience it directly. Alarm cues use the information that something else was injured to stay informed about a potential risk, while generalization allows for a better-informed initial response for novel risk. Safety generalization is important for preventing unnecessary risk associations, as shown in humans (Vervliet *et al.,* 2010), and could help to indicate when risk levels have dropped. Embryonic learning can also allow for learning about risk without experiencing it directly, such as learning about non-egg predators while still in the egg (Anderson & Brown, 2009; Warkentin, 2011). Embryonic information can also be generalized (Ferrari & Chivers, 2009a; Ferrari & Chivers, 2010; Ferrari & Chivers, 2011), although rarely are the gradients compared across developmental stages. The end result of generalization is tied to the information content of the initially exposed stimulus (Ferrari *et al.,* 2016), which might be altered based on the ontogenetic stage of the individual.

1.6: Purpose of Study:

My research was performed to determine whether exposure to an odour during post-neural fold formation affects the generalization gradient of wood frog tadpoles when compared to tadpoles exposed to the same odour during early post-hatch larval stages. Classical conditioning was used to train an odour under two contexts: risk association or latent inhibition. Alarm cues were used to establish risk and latent inhibition was used to establish safety. These served as the short-term events that are usually associated with resonance-type phenomena (Massot & Aragón, 2013; Ferrari *et al.,* 2019), and were used to construct the generalization gradient at a later developmental state. A phylogenetic gradient involving various species and hybrids of trout, whose phylogenetic relatedness is known, served as the generalization gradient (Murata *et al.,* 1993; Crespi & Fulton, 2004, Horreo, 2017). Post-neural fold formation around Gosner stage 12 (Gosner, 1960) in early ontogeny was used as the sensitive period. The well-established line cross metric was used as an indicator of activity levels (Ferrari *et al.,* 2007b), which always decrease for tadpoles when undergoing fear reactions. This study was designed to continue to integrate risk association, latent inhibition, and generalization through cognitive resonance, which itself is an integration of cognition, ecology, and animal behaviour research.

This thesis is split into four chapters. Chapter One presented the theoretical foundation of the thesis as well as provide a literature overview. Experiment One is presented in Chapter Two, which focuses on the effects of using risk association as the initial context to explore cognitive resonance and its effects on generalization gradients. The hypothesis is that the generalization gradient will be different for embryonically trained tadpoles than for larval trained tadpoles. The exact way the embryonic gradient will differ from the larval gradient depends on how the sensitive period affects the gradient and will be discussed in Chapter Two. Experiment Two is presented in Chapter Three, which focuses on the effects of using safety-related information association, through latent inhibition, as the initial context to explore cognitive resonance and its effects on generalization gradients. The hypothesis is that the generalization gradient will be different for embryonically trained tadpoles than for larval trained tadpoles. The exact way the embryonic gradient will differ from the larval gradient depends on how the sensitive period affects the gradient and will be discussed in Chapter Three. Finally, Chapter Four presents concluding remarks by comparing results from both experiments and speculating on future directions and consequences of this research.

Chapter 2: Cognitive Resonance and the Effects of Ontogenetic Development on the Antipredator Generalization Gradients in Wood Frog (*Lithobates sylvaticus*) Tadpoles

2.1: Abstract:

Generalization is a cognitive process that allows organisms to respond to novel stimuli without directly interacting with the source of the stimulus. This occurs through extrapolation of information from a known stimulus to a novel stimulus. Generalization is important for antipredator responses given an encounter with a predator may lead to death or injury. Antipredator responses through risk association can be maintained for longer if the first encounter occurs during early ontogeny. It is unknown if the timing of the first exposure to risk association also effects the generalization gradient for antipredator responses. This study addresses this gap in knowledge by using wood frog tadpoles to compare the ability to generalize between groups first taught a predator at different life stages. Tadpoles were first taught that brook trout was a predator as either embryos or larvae and then tested for recognition to brook trout, the hybrid species splake or tiger trout, the closely related rainbow trout, or the distantly related goldfish. Embryonically taught tadpoles recognized every trout test odour as a predator due to brook trout training, whereas larval taught tadpoles only recognized the brook trout hybrid species. This study provides evidence that early development can influence the shape of generalization gradients.

2.2: Introduction:

Alarm cues are an informative indicator for predation risk, since the suite of odours that make up alarm cues are only released upon the injury of a conspecific (Chivers & Smith, 1998). Since the individual that releases the alarm cues is severely harmed in the process, the odds of release outside of risky environments is small. Alarm cues are a classic example of a US for risk and are informative enough that a single pairing with a CS is often enough to generate an antipredator response (Suboski, 1990; Ferrari *et al.,* 2010b; Gonzalo *et al.,* 2010). Alarm cues are a commonly used risk indicator in wood frog studies (Chivers & Mirza, 2001; Ferrari & Chivers, 2009a; Ferrari *et al.,* 2010a; Chivers & Ferrari*,* 2013; Chivers *et al.,* 2015; Ferrari *et al.,* 2016). Associative learning using alarm cues is required for wood frog tadpoles to learn of predation threats as wood frog tadpoles do not innately recognize many fish (Ferrari *et al.,* 2010a; Chivers & Ferrari*,* 2013; Chivers *et al.,* 2015; Ferrari *et al.,* 2016), amphibians (Ferrari *et al.,* 2009; Ferrari & Chivers, 2009a; Ferrari *et al.,* 2010a; Chivers & Ferrari, 2013), or dragonfly larvae (Chivers & Mirza, 2001) as risky.

While alarm cues are an informative indicator of risk, responses to alarm cues can be altered based on the perception of the true level of risk. One such method is neophobia, which is the expression of risk responses in the presence of ambiguous or novel stimuli (as first described by Barnett, 1958). Pre-exposure to unpaired alarm cues is known to induce neophobia in aquatic systems (Brown *et al.,* 2013). This is because the lack of an association made between the alarm cues and a neutral stimulus indicates that anything in the environment might be the source of risk. Other sources of risk information can also provide crucial context to help enhance risk responses, such as diet cues (Mathis & Smith, 1993), disturbance cues (see Bairos-Novak *et al.,* 2017 for review), and social cues in fish (Crane *et al.,* 2015).

The developmental timing of the first exposure can also alter the perception of risk related information. Ferrari *et al.* (2019) showed that tadpoles which had undergone embryonic learning managed to maintain an antipredator response for a longer period of time when compared to tadpoles that learned the same information at a later point in time, without changing the intensity of the response. This cognitive resonance used the disproportionate influence caused by sensitive periods to achieve the resonance effect. Sensitive periods are periods of time when the current environment has a disproportionate effect on phenotype compared to later stages, which are expected to occur when uncertainty about the environment is greatest (Fawcett

& Frankenhuis, 2015; Fuhrmann *et al.,* 2015). It is known that events during sensitive periods can have greater effects later in life on both behavioural phenotypes and cognitive abilities (Massot & Aragón, 2013; Whiteside *et al.,* 2016).

Early development is an example of a sensitive period since individuals have no prior information about their current environment, so sampling has the potential to rapidly reduce uncertainty (Fawcett & Frankenhuis, 2015). Wood frog tadpoles can learn predation-related information as embryos, as was first demonstrated using red-bellied newts (Mathis *et al.,* 2008). For olfactory information, this sensitive period might be as early as Gosner stage 12, when the neural fold begins to form, or closely thereafter (Supekar & Gramapurohit, 2017). Embryonic learning has a clear fitness benefit for aquatic prey species, as it may allow them to learn about possible future predators in their environment. Since not every predator that eats tadpoles will eat the egg stages (Anderson & Brown, 2009), tadpole embryos can delay hatching in order to avoid a temporary larval predator or hatch sooner to prevent predation by an egg predator, although the degree to which this plays a role in aquatic amphibian systems is still debated (Warkentin, 2011)

Tadpoles can use information obtained through alarm cues to extrapolate beyond the initial conditions. One method that tadpoles can use is to extrapolate the changes in risk-levels to recognize and respond to overall trends in risk (Crane & Ferrari, 2017). A sudden increase in risk will result in a greater antipredator response than a linear increase in risk and a linear increase in risk will result in a greater antipredator response than a constantly high risk, even if the ending level of risk is the same for all three. Tadpoles can also extrapolate risk information to novel stimuli using generalization. Generalization is the cognitive process by which the learned response of one stimulus is applied to a novel stimulus (see Ghirlanda & Enquist, 2003). The relation between the similarity of the response and the degree of similarity, when using multiple novel stimuli, forms a generalization gradient (Kalish & Guttman, 1957). Generalization has been studied using predation information (Griffin *et al.,* 2001; Smith *et al.,* 2008; Davis *et al.,* 2012; Chivers & Ferrari, 2013), specifically through alarm cues (Ferrari *et al.,* 2007a; Ferrari *et al.,* 2009; Ferrari & Chivers, 2009a; Ferrari & Chivers, 2010; Chivers *et al*., 2016; Ferrari *et al.,* 2016). In wood frog tadpoles, the phylogenetic distance between the potential predators is often used as the degree of similarity to serve as the basis of generalization for olfactory information (Ferrari & Chivers, 2009a).

There are factors already known to influence the shape of generalization gradients in wood frog tadpoles and other aquatic prey species, such as time (Chivers & Ferrari, 2013; Ferrari *et al.,* 2019), information quality (Chivers *et al.,* 2015; Ferrari *et al.,* 2016), and background exposures (Ferrari & Chivers, 2009a; Chivers *et al.,* 2016). Two such alterations of note are steepening and flattening. Steepening occurs when responses to stimuli that are more similar to the initial stimulus increase and responses to stimuli that are less similar to the initial stimulus decrease (Hearst & Poppen, 1965). Steepening is known to happen when the overall quality of the information learned increases when using operant conditioning paradigms (Farthing & Hearst, 1968), although alarm cues are a classical conditioning paradigm. Sensitive periods might cause a steepening of the generalization gradient, as the information encountered during the sensitive period is predicted to have the highest capacity to reduce uncertainty (see Fawcett & Frankenhuis, 2015 for a review).

Flattening occurs when the responses to stimuli further from the initial stimulus increase and responses closer to the initial stimulus decrease (Bouton *et al.,* 1999), which is known to happen as time between training and testing of an odour increases (Gil *et al.,* 2017). Embryonic learning might result in flattening of the generalization gradient if the only factor influencing the generalization gradient is time between training and testing, as there is more time to forget the non-reinforced information. Flattening might also occur due to differences in neurological and morphological development as a result of staging index (Gosner, 1960), as the olfactory system is not as developed in embryonic wood frogs compared to larval wood frogs. The lack of development does not prevent other anurans from learning risk-related information as shown in the skipper frog *Euphlyctis cyanophlyctis* (Supekar & Gramapurohit, 2017), which can learn predation related information at Gosner stage 12, the gastrula stage before the neural plate forms. Only if presented continuously until Gosner stage 33 was the response significantly stronger than the tadpoles taught at Gosner stage 12, indicating an age-related alteration in information quality while also following morphological and neurological development. The protective jelly layer and capsule layers might not be permeable to some odour components (Pinder & Friet, 1994), further altering information content.

The generalization gradient might also alter in a multi-step process or in a way that is explainable through multiple mechanisms working concurrently. Ferrari *et al.* (2016) suggested a multi-step method towards alterations of generalization gradients. Tadpoles with higher

quality information undergoing a steepening-like phenomenon, called sharpening, for odours beyond a certain relatedness point to the initial stimulus. Tadpoles also underwent a flattening-like phenomenon called broadening for odours closely related to the initial stimulus, which was then followed by a rapid decrease in responding to increasingly distant stimuli referred to as sharpening. This was speculated to be because uncertain information needs to be applied more broadly due to the potential for error in receiving, encoding, storing, or retrieving the information. Certain information has less chance for error and tadpoles could be more certain that related stimuli would have related impacts on the individual. It is also possible that the shape of the gradient does not change but is retained for longer. In Ferrari *et al.,* (2019), predation related responses were maintained for 11 days post conditioning for embryonically exposed tadpoles but were lost in larval exposed tadpoles after 11 days. Generalization gradients could be affected the same way due to shared cognitive resources, such as relying on the same stored information.

In this study, the effects of cognitive resonance during early development on the alteration of antipredator generalization gradients were explored. This was done by asking the following four questions: 1) Can embryonically exposed tadpoles form a generalization gradient from risk associated cues with brook trout as the initial odour? 2) What does the generalization gradient look like? 3) Is the generalization gradient different from the gradient formed by larval exposed tadpoles? 4) In what way do the generalization gradients different? There are multiple predictions on exactly how the gradient will change and there is no theoretical backing to prefer one over the others. Flattening is expected to occur in the embryonically trained tadpole generalization gradient if the sensitive period has little additional effect and time is the only factor. But flattening is expected to happen in the larval trained tadpole generalization gradient if the embryonically obtained information has enough reduced forgetting to slow the rate of flattening in the embryonically exposed tadpoles. Steepening is expected to occur in the embryonically trained tadpole generalization gradient if embryonic information is perceived as more informative, while the larval trained tadpole generalization gradients should steepen if more recent information is perceived as more relevant. Finally, it is possible that both processes interact, generating a concurrent broadening and sharpening phenomenon in one of the groups of tadpoles.

2.3: Methods:

2.3.1: Experimental Overview:

In order to test the effects of sensitive periods on the generalization gradient in tadpoles, a fully crossed 2 x 2 x 5 factorial experiment was designed with the following factors: ontogeny (two levels: embryonic and larval), conditioning (two levels: brook trout predation pre-exposure and control), and cue (five levels: brook trout, splake, tiger trout, rainbow trout, and goldfish). Brook trout (*Salvelinus fontinalis*) was the conditioned predator odour. Fish used to construct the generalization gradient were brook trout (*Salvelinus fontinalis*), splake (*Salvelinus fontinalis x Salvelinus namaycush*), tiger trout (*Salvelinus fontinalis x Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), and goldfish (*Carassius auratus*). These species were chosen to maximize the *a priori* knowledge of the phylogenetic relatedness between the test species in order to have an unambiguous phylogenetic gradient. Splake is a hybrid between the conditioned species odour and member of the same genus, lake trout (*Salvelinus namaycush*). Tiger trout is a hybrid between the conditioned species and a member of different genus, brown trout (*Salmo trutta*). Rainbow trout is a member of a different genus but within the same family (Salmonidae). Goldfish is a member of a different family (Cyprinidae). Species chosen for the test odours have a known phylogenetic relation (Murata *et al.,* 1993; Crespi & Fulton, 2004, Horreo, 2017). Freezing behaviour was chosen as the most reliable and easily measured of the antipredator responses in tadpoles.

2.3.2: Test Species:

Wood frog eggs (*Lithobates sylvaticus*) were collected in Alberta between the end of April and the beginning of May and reared until hatching (~five days). The first tadpoles collected were used as donors for alarm cues, while the rest were experimental tadpoles. They were fed alfalfa pellets every 2-3 days post-hatch as an additional food source in conjunction with algae that was already present in the holding tank.

2.3.3: Cue Preparation:

2.3.3.1: Alarm cues: Eggs were collected at the end of April 2018 in Alberta. These eggs were reared until the tadpoles reached an approximate size of 10 mm. Preparation of alarm cues followed a well-established procedure (Mathis *et al.,* 2008; Ferrari & Chivers, 2009a). Each tadpole was euthanized with a blow to the head and ground up using a mortal and pestle.

The combined crushed tadpoles were diluted with well water until the final dilution was three tadpoles per two liters of water. The well water was exposed to the external environment for a minimum of 12 hours due ensure the gaseous solutes in the water closely matched that of the atmosphere. Alarm cues were made as needed on the day of conditioning.

2.3.3.2: Test Odour: All fish were housed in the RJF Smith building of the University of Saskatchewan. Trout were fed once a day with a pellet diet and goldfish were fed once a day with a commercial flake food diet. Tanks of water (50 L for trout and 10 L for goldfish) were set out 24 hours prior to the addition of the individual fish in order for the temperature of the water to stabilize at 19 C. All tanks were set up with an air stone but no filter. Individual brook trout (mean total length ± SD = 30.5 ± 0.8 cm), splake (mean total length ± SD = 34.2 ± 0.6 cm), tiger trout (mean total length ± SD = 35.7 ± 1.8 cm), and rainbow trout (mean total length ± SD = 38.6 ± 3.4 cm) were then placed into the 50 L tanks for a 24-hour period. Goldfish (mean total length ± SD = 14.3 ± 1.6 cm) were placed into the 10 L tanks for a 24-hour period. During this period, fish were not fed to decrease the influence of diet cues (Mathis & Smith, 1993). Two individuals of each species were isolated at the same time in order to prevent individual level differences in odour from affecting the results. After the 24-hour period, fish were removed from the tanks. Water was extracted from both tanks of any given species and was mixed together. Samples were then frozen at -20 C until further use.

2.3.4: Treatments:

Six clutches of eggs were collected and each clutch was split into four equally sized sub-clutches 24-hours post-collection. Each sub-clutch was assigned to a 7 L pail (24 pails in total) containing 2 L of well water. Pails were arranged in six columns and four rows. Each clutch occupied a single column to account for slight environmental gradients. Eggs were left in the pail for two days. On day two post-fertilization (May 9th, 2018), pails assigned to the embryonic predator odour (PO) and alarm cues (AC) treatment were conditioned with 20 mL of brook trout odour immediately followed by 10 mL of alarm cues, while tadpoles assigned to the embryonic PO+water control treatment were conditioned with 20 mL of brook trout odour immediately followed by 10 mL of well water. Eggs in the larval treatments were conditioned with 30 mL of well water to maintain disturbance regime. This was done twice daily over the course of two days, with full water changes occurring after the second conditioning on each day. A water change was performed four hours after the last conditioning of the day. On day ten post-

fertilization (May 17th, 2018), tadpoles assigned to the larval PO+AC treatment were conditioned with 20 mL of brook trout odour followed by 10 mL of alarm cues, while tadpoles in the larval PO+water control treatment were conditioned with 20 mL of brook trout odour immediately followed by 10 mL of well water. Tadpoles that had already received the embryonic treatments were conditioned with 30 mL of well water to maintain disturbance regime. This was done twice daily over the course of two days. A water change was performed four hours after the last conditioning of the day.

2.3.5: Testing:

Testing procedures followed well established protocols using the decrease in activity that tadpoles perform when under predation risk (Chivers & Mirza, 2001; Ferrari *et al.,* 2007b; Ferrari *et al.,* 2007c; Mathis *et al.,* 2008; Ferrari & Chivers, 2009a). All testing occurred one week after the larval conditioning (May 25th and May 26th, 2018). Tadpoles were tested in four rounds over the course of two days. Trays containing three rows of five cups were laid out in the open away from shadows that might cause a disturbance in the tadpoles. Cups were assigned a place number 1-5, which corresponded to a test odour number. All cups in the same column received the same test odour. Odours were randomly assigned to a column. Each row contained tadpoles from one pail, and pails were chosen such that a tray would contain mostly tadpoles from the same clutch. The pre-treatment for each pail and the test odour were both unknown to those performing the experiment. Five tadpoles from each pail were selected at random and brought to the location of the trays. One tadpole was assigned to each cup and was given approximately 45 minutes to acclimate to the conditions of the cup. Observers were then stationed at each tray and watched each cup. For four minutes pre-test, observers counted the number of line crosses each tadpole made across an imagined line through the middle of the cup to serve as a baseline. A line cross was defined as when the entire body of the tadpole switched from one side of the cup to the other. Any tadpole that did not have a pre-test baseline line cross of ten or greater was considered inactive and not used in the analysis. Observers then added 5 mL of the assigned test stimulus into each cup over a period of 45 seconds. After the injection period, a four-minute post-test line cross observation period was performed.

To make sure the treatments did not cause a difference in overall body size, a subsample of tadpoles was measured between 0-42 hours post-observation period. While not an expected outcome, it is possible that exposure to alarm cue during one stage results in one conditioning

treatment eating less than the other. Even a short behavioural alteration can result in the same suite of behavioural and body size changes that were noted in the foundational paper on phenotypic resonance (Massot & Aragón, 2013). The length of each tadpole was measured from head to tail. Measurements could not be linked directly to the individual tadpole, only to the tested odour and pail number.

2.3.6: Statistical Analysis:

An activity metric was defined as: $(Post - Pre)/Pre$ and used for all behavioural statistical analyses. A prior analysis using pre-injection activity and removing only tadpoles with pre-injection line crosses less than 10 (1 case) revealed a significant effect of conditioning ($F_{1,14.3} = 5.883, p = 0.029$) with the controls showing lower activity, indicating a need to use proportional change in line crosses. The data were analyzed as a 5-way nested ANOVA using a Type I Sum of Squares to account for the nested factors in the design, since Type I Sum of Squares accounts for the order the terms are presented while. Conditioning treatment, ontogeny treatment, and tested cue were fixed factors. Clutch was included as a blocking factor. Pail was included as a nested factor to account for the non-independence of some of the tadpole observations as each conditioning was applied to a pail containing multiple tadpoles that were subsequently tested. The model included all fixed main effects first, followed by all fixed 2-way interactions, then the fixed effect 3-way interaction, then clutch, and then pail nesting ontogeny*conditioning. Any data with a pre-injection line cross of less than 10 that were accidentally recorded (1 case) were removed from the data set. Any data with a proportional change in line crosses greater than 1.0 (5 cases) were also removed from the data set. This was done to have the maximum proportional difference match the lower bound for the minimum proportional difference at -1.0. A boxplot analysis was performed using Tukey's hinges and all outliers greater than 1.5*IQR+Q3 or less than 1.5*IQR-Q1 (45 cases) were removed from the data set. A Levene's Test for heterogeneity of variances was run to test for the assumption of equal variances. Residual plots were examined to test for the assumption of normally distributed residuals. The interaction between clutch and all fixed factors was run with the original model using a separate ANOVA to test the assumption of non-interaction between the blocking factor and treatments. All significant interactions were analyzed by splitting the data set along one of the main factors within the significant interaction and using subsequent nested ANOVAs and Tukey's HSD post-hoc tests. All remaining data points were analyzed (N=582) at $\alpha = 0.05$.

Size data were analyzed in a separate 5-way nested ANOVA using a Type I sum of squares with conditioning, ontogeny, and tested cue as fixed factors and with clutch included as a blocking factor. Pail was included as a nested factor to match the ANOVA run on the behavioural data. No data points were removed for this analysis (N = 233). All statistical analyses were done in SPSS V.25 and all graphs were made in Microsoft Excel 2017.

2.4: Results:

An inspection of the residual plots for the proportional change in line crosses did not show an overall deviation from normality. A Levene's test revealed no significant heterogeneity. There was no significant interaction between clutch and the treatment groups. There was no significant effect of pail or clutch. There was no significant effect of clutch on size. There was a significant effect of pail on size ($F_{15,198} = 1.905$, p = 0.025), but no other significant interactions with pail were detected. A significant three-way interaction between ontogeny*conditioning*cue ($F_{4,538.9} = 2.877$, p = 0.022) was revealed. The data were split by conditioning and a 4-way ANOVA was performed. Ontogeny and cue were kept as fixed factors, while pail was kept as a nested factor and clutch was kept as a blocking factor. In the brook trout control group, a significant effect of ontogeny was found ($F_{1,4.4} = 9.335$, p = 0.033) with the embryonic control group showing a greater proportional change in activity compared to the larval control group (Fig. 2.1). No significant effect was found for cue ($F_{1,4.4} = 0.22$, p = 0.93) or for the ontogeny*cue interaction ($F_{4,246.2} = 0.61$, p = 0.66) between the control groups. A significant effect of ontogeny*cue in the brook trout + alarm cues treatment was revealed ($F_{4,292.8} = 3.592$, p = 0.007). The brook trout + alarm cue data set were split by ontogeny and analyzed with a 2-way ANOVA. Cue was kept as a fixed factor, while clutch was kept as a blocking factor. Pail was removed as a nested factor since pail nested ontogeny*conditioning which was an interaction that could not be run when the ANOVA was split by both ontogeny and conditioning. A significant effect of test cue was found in both the larval ($F_{4,143.6} = 16.08$, p < 0.001) and embryonic ($F_{4,148.9} = 19.49$, p < 0.001) treatment groups. A Tukey HSD post-hoc analysis revealed significant pairwise differences between the pairwise groups (Fig. 2.2).

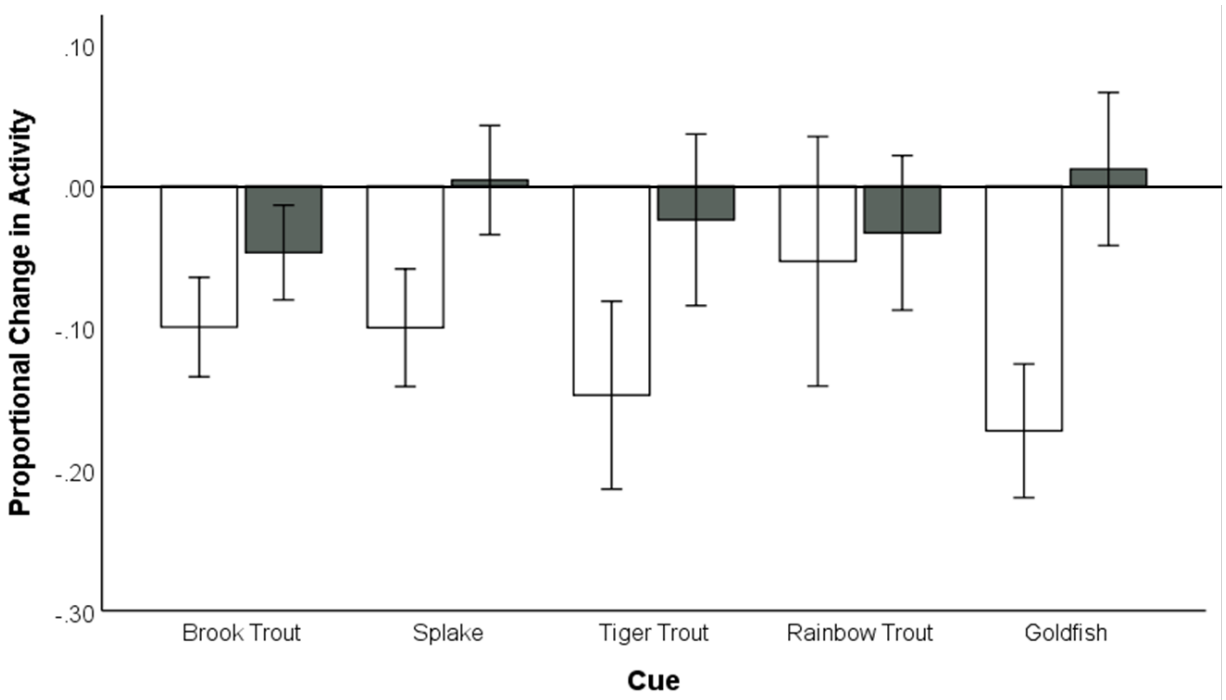

Fig. 2.1.

Mean proportional decrease in line crosses (± SE). White bars indicate embryonically exposed brook trout + water treatment tested with brook trout (n=23), splake (n=23), tiger trout (n=26), rainbow trout (n=30), or goldfish odour (n=22). Dark gray bars indicate larval exposed brook trout + water tested with brook trout (n=22), splake (n=30), tiger trout (n=33), rainbow trout (n=28), or goldfish (n=27) odour. No significant differences were found using Tukey's HSD. Significant differences in the ontogeny main effect were found using an F-test ($\alpha = 0.05$).

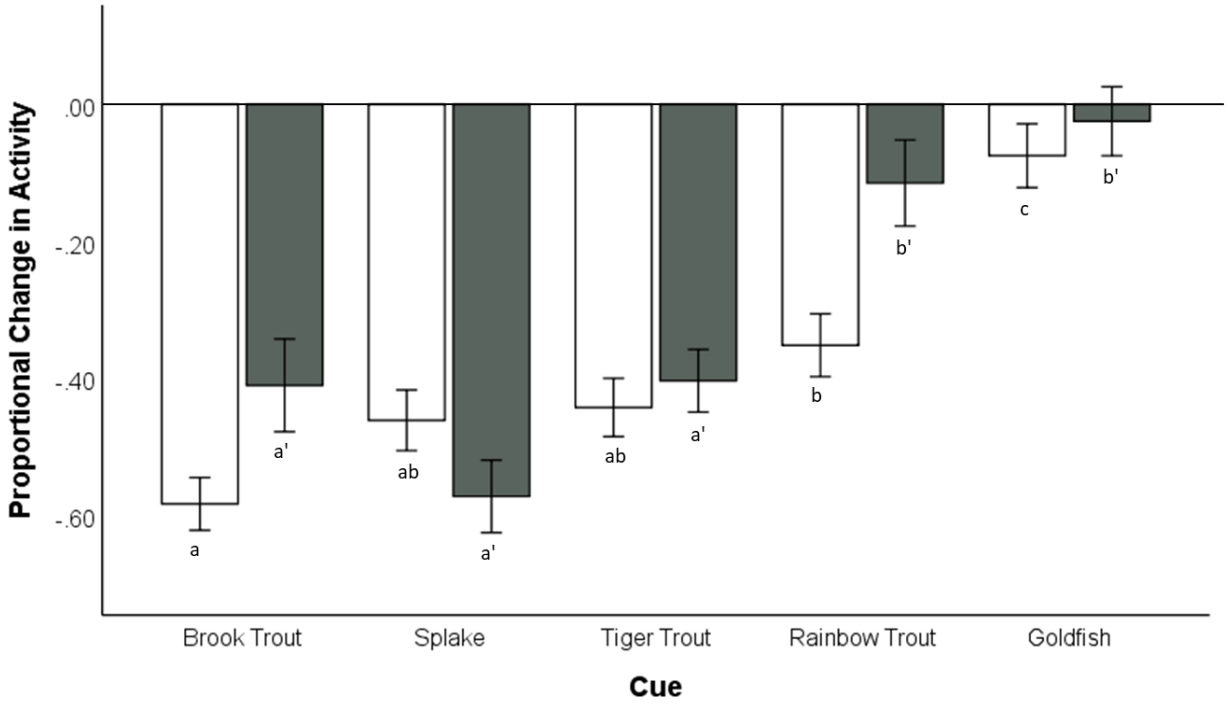

Fig. 2.2.

Mean proportional decrease in line crosses (± SE). White bars indicate embryonically exposed brook trout + AC treatment tested with brook trout (n=33), splake (n=30), tiger trout (n=33), rainbow trout (n=31), or goldfish odour (n=31). Dark gray bars indicate larval exposed brook trout + alarm cues treatment (AC) tested with brook trout (n=29), splake (n=27), tiger trout (n=31), rainbow trout (n=34), or goldfish (n=29) odour. Letters indicate significant differences using Tukey's HSD ($\alpha = 0.05$).

2.5: Discussion:

This study provides evidence that the olfactory risk-associated generalization gradient is affected by the timing of the first exposure in wood frog tadpoles. Embryonically exposed tadpoles (or EAC for embryonic alarm cues) responded to rainbow trout with statistically the same intensity as splake and tiger trout, each of which showed statistically the same intensity as the risk trained brook trout, whereas larval exposed tadpoles (or LAC for larval alarm cues) did not (Fig. 2.2). The response to rainbow trout in the EAC tadpoles was lower than the response to brook trout but higher than the control response. As such, EAC tadpoles can distinguish rainbow trout from brook trout enough to prevent responding to rainbow trout with the same intensity as brook trout, but EAC tadpoles still consider rainbow trout similar enough to brook trout to respond to rainbow trout as potentially dangerous. Conversely, LAC tadpoles do not respond to rainbow trout as a potential predator. Only brook trout, splake, and tiger trout are recognized as potential predators; where splake and tiger trout are hybrid species formed by brook trout males mating with other trout species. It is also worth noting that the embryonic control tadpoles (or EWC for embryonic water control) did show a greater antipredator response in general in comparison to larval control tadpoles (or LWC for larval water control), as seen in the control treatment comparisons (Fig. 2.1). It could be that tadpoles exposed to any odour as embryos are more sensitive to disturbances than tadpoles exposed to any odour as larvae, although the fact that the EAC tadpoles showed no neophobic reaction to the goldfish indicates that this sensitivity plays a minor role and only serves to increase the baseline response for EAC tadpoles than LAC tadpoles.

The non-response of LAC tadpoles to rainbow trout at first appears at odds with some previous experiments involving tadpole generalization (Chivers *et al.,* 2015, Chivers *et al.,* 2016, Ferrari *et al.,* 2016), which showed larval exposed tadpoles as capable of generalizing between rainbow trout and brook trout. In Chivers *et al.,* (2016) and Ferrari *et al.* (2016), this was done by generalizing rainbow trout to brook trout, while in Chivers *et al.* (2015), this was done by generalizing tiger trout to rainbow trout and brook trout. In both cases, the instances with the greatest degree of generalization between the two involved exposing tadpoles to one pairing of alarm cues and predator odour, whereas this experiment used four pairings. This makes the current study more comparable to the high-quality treatment group in Ferrari *et al.* (2016), where the high-quality treatment group was exposed to rainbow trout paired with alarm cues for five

conditioning groups. The current study follows a similar methodology, except with one less conditioning and performed over two days instead of three days. In Ferrari *et al.* (2016), the high-quality treatment group still responded to brook trout when compared to goldfish, though far less than the once conditioned low-quality group. It could be that information contained in rainbow trout odour allows for easier generalization into brook trout than the reverse. Despite this slight discrepancy, the relative lack of response between rainbow trout and brook trout in both this study and in Ferrari *et al.* (2016) means that the two are not completely at odds.

The generalization gradient did not appear to undergo strict steepening or flattening. The decrease in response to the less related rainbow trout (Crespi & Fulton, 2004, Crête-Lafrenière *et al.,* 2012) in the LAC tadpoles would at first appear to support steepening, but steepening usually involves an increase in responsiveness to stimuli around the initial stimulus and is usually only applied to stimuli that can vary in both directions along a given stimulus axis (Hearst & Poppen, 1965), neither of which occurred. The currently known effect of information quality on wood frog tadpole generalization gradients is mixed, with some studies indicating an alteration akin to steepening (Ferrari *et al.,* 2016) and others not showing any change (Chivers *et al.,* 2016). It could also be that the EAC generalization gradient is flattening compared to the LAC generalization gradient due to the increased time between the final conditioning and testing. This could be a likely explanation since flattening usually involves an increase in responsiveness to more distant stimuli (Gil *et al.,* 2017). However, the maintenance of the response to the initial stimulus and the fact that the stimulus could not vary in both directions along the stimulus axis suggests that flattening is likely not the major phenomenon for differences in the generalization gradients presented in this study (Bouton *et al.,* 1999). To completely rule this out, it would have to be demonstrated that the two durations used do not result in the same gradient relationship when the same experiment is performed outside of the potential sensitive period.

The generalization gradient changes appear to match more closely to either a multi-step alteration in the LAC tadpoles or a resistance to flattening in the EAC tadpoles. The multi-step alteration would follow from the broadening and sharpening of the generalization gradient found in Ferrari *et al.,* (2016). Analogies can be drawn between the EAC tadpoles and the uncertain tadpoles, as well as the LAC and the certain tadpoles, in Ferrari *et al.* (2016). It appears that the EAC tadpoles broadened the range of recognizably risky stimuli along a gradient, while the LAC appeared to create more distinct categories. The odours that induced a response in the LAC

tadpoles all roughly matched, while odours that were recognized in the EAC tadpoles existed on more of a gradient. This matches well with the predictions set out by Ferrari *et al.* (2016), implying the cause of the change to the generalization gradient was the alternation in the perceived quality of the CS, but not in such a way to cause steepening.

It is also still possible that the generalization gradient did not change but was rather maintained longer in the EAC tadpoles rather than the LAC tadpoles, as the EAC generalization gradient appears far more similar to prior larval generalization gradients (Chivers *et al.,* 2015, Chivers *et al.,* 2016, Ferrari *et al.,* 2016) than the LAC gradient generated by this study. Adding to that, in Chivers *et al.* (2015) and Ferrari *et al.* (2016), tadpoles were tested one day after the last exposure, whereas tadpoles in this study were tested one week after the last exposure. The time differential may explain why the larval generalization gradient looks different, as the information is being forgotten. However, Chivers *et al.* (2016) had a 9-day waiting period before testing and still found a generalization gradient that included rainbow trout and brook trout. More studies need to be done to ascertain whether EAC tadpoles are maintaining the same generalization gradient for a longer period of time than LAC tadpoles, such as testing the generalization gradients right after larval exposure and seeing if that generalization gradient matches the embryonic gradient. The decay of the generalization gradients for both embryonically exposed and larval exposed tadpoles can also be explored by performing the same setup but testing tadpoles at multiple time intervals.

If the above pattern holds for the EAC tadpole generalization gradient and the LAC tadpole generalization gradient, the EAC tadpoles should be able to generalize to more trout species than LAC tadpoles. EAC tadpoles generalized from brook trout to rainbow trout, whereas LAC tadpoles did not. According to current phylogenies (Crespi & Fulton, 2004, Crête-Lafrenière *et al.,* 2012), brook trout and lake trout are within genus *Salvelinus* (splake is a *Salvelinus* intra-genus hybrid). *Salvelinus* and rainbow trout (*Oncorhynchus*) are part of a larger subdivision within Salmonidae, while brown trout is more distantly related (tiger trout is an inter-genus hybrid). Since the LAC tadpoles are only generalizing to trout that contain brook trout as one of the parental species, the number of trout species that LAC tadpoles can generalize to is expected to be limited to brook trout or perhaps smaller taxonomic divisions containing members of *Salvelinus*. Since the EAC tadpoles generalized to rainbow trout, it is expected that this generalization gradient would extend to other species within this greater *Salvelinus*-

Oncorhynchus subdivision of Salmonidae. Future studies should use the generalization gradient produced from this and other such studies to see if the gradients can predict responses to other species with different levels of relatedness within the *Salvelinus-Oncorhynchus* block (Horreo, 2017), such as Sockeye salmon (*Oncorhynchus nerka*), Chinook salmon (*Oncorhynchus tshawytscha*), or the Arctic char (*Salvelinus alpinus*).

The ability for EAC tadpoles to generalize to more potential predatory species provides a clear fitness advantage for tadpoles exposed to predation related information early on. These tadpoles will be able to learn about novel predator faster than LAC tadpoles or maintain that response for longer than LAC tadpoles. This should provide a survival related fitness benefit to the tadpoles as phylogenetically related species often have similar ecological niches to each other (Losos, 2008). This is especially important if the tadpoles must respond to an invasive species, as demonstrated by the Iberian water frog *Pelophylax perezi* (Nunes *et al.,* 2014) and wood frog (Ferrari *et al*., 2015). Alternatively, the generalization gradient for the EAC tadpoles could come at a fitness cost due to the added time and energy spent on antipredator responses as would be predicted by the Risk Allocation Hypothesis (Lima & Bednekoff, 1999). While a properly generalized threat response is adaptive, a false positive assigned to a non-risky stimulus results in energetically costly avoidance behaviour being chosen over foraging. Such unnecessary responses would give the fitness advantage to the LAC tadpoles over the EAC tadpoles through increased resource acquisition in the event that the distantly related novel odour is different enough from the initial odour to not pose a threat. Failure to gather enough resources before metamorphosis could result in the tadpole dying or affect when metamorphosis occurs (Crump, 1981). While maintaining a predation generalization gradient for longer has some clear advantages, it can be disadvantageous if the process of generalization results in too many false positives.

Chapter 3: Cognitive Resonance and the Effects of Ontogenetic Development on the Generalization Gradients of Latently Inhibited Cues in Wood Frog (*Lithobates sylvaticus*) Tadpoles

3.1: Abstract:

Latent inhibition is a mechanism that organisms use to learn about safety in their environments through repeated exposures to a neutral stimulus until different associations for that stimulus become difficult to form. Safety information can be generalized and applied outside the original stimulus or context, which can aid in proper excitatory responses. Cognitive resonance is a recently described phenomenon whereby cognitive information presented during a sensitive period has a disproportionate effect on the interpretation of information and other cognitive processes later in life, but it is unknown if cognitive resonance applies to latent inhibition and generalization. To investigate this phenomenon, wood frog tadpoles were pre-exposed to brook trout as either embryos or larvae in order to latently inhibit the stimulus. Then a second conditioning was performed using one of five fish odours along a phylogenetic gradient to train tadpoles that the fish odor was risky. The antipredator reactions to this fish odour were tested using a line cross metric. The study indicates that larval tadpoles generalized to the intra-genus hybrids, while embryonic tadpoles had a shallower gradient that generalized to the other trout species. This indicates that cognitive resonance occurred by altering the shape of the generalization gradient and that sensitive periods can have consequences for inhibitory cognitive processes later in life.

3.2: Introduction:

Latent inhibition is a classical conditioning phenomenon that decreases ease of pairing a known neutral stimulus to a novel stimulus, as described by Lubow & Moore (1959). Latent inhibition is done by repeatedly exposing an individual to a novel stimulus until the stimulus becomes familiar or attentional resources are no longer diverted to it (see Lubow, 1973 for review). Once a stimulus has undergone latent inhibition, the formation of any novel association becomes increasingly difficult, usually requiring a lengthy reversal learning period or temporal extinction period (reviewed by Bouton, 2004). The reversal learning process usually requires an excitatory stimulus to be paired with the inhibited stimulus to see if the new association has been prevented (Kraemer & Golding, 1997; see also Hazlett, 2003 for crayfish example and Ferrari & Chivers, 2006 for minnow example). In aquatic systems, this is usually accomplished using alarm cues (Ferrari & Chivers, 2008; Ferrari & Chivers, 2011; Mitchell *et al.,* 2011; Gonzalo *et al.,* 2013).

Latent inhibition is an inhibitory classical conditioning phenomenon (Pavlov & Gantt, 1928; Rescorla, 1969), which includes the learned safety paradigm of conditioned inhibition (Rescorla & Wagner, 1972; Pollak *et al.,* 2010). Latent inhibition is mechanistically the same as the retardation test, one of the two requirements to determine if a conditioned inhibitor has been learned as safe (Rescorla, 1971; Savastano *et al.,* 1999), although they are not the same process due to the different methods of initial inhibition. Latent inhibition in psychology and animal cognition are also different from each other, as latent inhibition in psychology is tested by inhibiting irrelevant stimuli during a task (Lubow, 2005) while latent inhibition in animal cognition uses pre-exposure to a neutral stimulus (Ferrari & Chivers, 2006). However, the two processes are similar enough to allow for animal models to serve as proxies for human latent inhibition (Lubow, 2005). Latent inhibition has also been used in aquatic systems as an ecologically relevant proxy for safety (Ferrari & Chivers, 2011).

Background information is expected to affect latent inhibition learning and behavioural responses. One such way is through the Risk Allocation Hypothesis (Lima & Bednekoff, 1999), which states that individuals must balance the energetic demands of antipredator responses with the need to obtain food. As such, the amount of antipredator responses should depend on the frequency of risk indicators/encounters and the proportion of time spent in a risky environment. The degree of antipredator responses in a safe environment shows a greater range of variability

than does the degree of antipredator responses spent when the environment is risky. It would be beneficial for an individual to be able to actively sample for safety related cues in order for it to alter its antipredator responses faster than relying on risk cues alone. However, these cues should be restricted in scope due to the asymmetric relation between safety related false positives and risk related false positives (Wiley, 1994 through Lynn *et al.,* 2005). If a stimulus is perceived as risky when it is safe, then energy is spent on antipredator responses; but if a stimulus is perceived as safe when it is risky, then the individual risks death. In Brown *et al.* (2015), it took longer for fish in a high-risk background to ignore the background cue in a similar process to latent inhibition. If the environment is inherently risky, then any novel cue is more likely to represent a risk. Similarly, in Chivers *et al.* (2014), high background risk prevented the latent inhibition directly in damselfish.

The interpretation of background information itself can change depending on when the information is first presented. Sensitive periods are stages of ontogeny when the individual is disproportionately influenced by its current environment compared to encountering the same environment at a later state (Bateson, 1979; Fawcett & Frankenhuis, 2015). The most common example of a sensitive period is early ontogeny due to the fact that early development is the first time an individual encounters environmental information and so is the first time an individual can alter its behaviour or phenotype (see Fawcett & Frankenhuis, 2015 for review). Olfactory information is a good sensory modality to use for studying sensitive periods as it is one of the first sensory systems to functionally develop in tadpoles (Graziadei & Monti-Graziadei, 1992; Hepper & Waldman, 1992). In Supekar & Gramapurohit (2017), it was demonstrated to embryonic tadpoles can learn olfactory cues before the development of the neural plate at Gosner stage 13 (Gosner, 1960), indicating a sensitive period that should occur before hatching.

Ferrari *et al.* (2019) showed that manipulating cognitive phenomenon, including latent inhibition, during a sensitive period can lead to cognitive effects later in life, in a phenomenon referred to as a cognitive resonance. Three reversal learning trials were enough to teach larval taught tadpoles that a latently inhibited cue was risky, but embryonically exposed tadpoles did not learn the latently inhibited cue was risky after the same number of exposures. This discrepancy could prove adaptive if the safety indicators remain safe throughout the relevant life stage, but might also prevent the tadpole from using the most recent and relevant information about its environment the environment happens to have changed. Holding on to irrelevant safety

information could be detrimental if a predator switches its preferred ontogenetic stage for predation efforts, leading to multiple pre-exposures before the predator could register as a risk for the prey species (as speculated in Ferrari & Chivers, 2011 and Mitchell *et al.,* 2011).

Generalization of latent inhibition is known to occur in animals (Siegal, 1969; Brown *et al.,* 2011; Ferrari & Chivers, 2011; Chivers *et al.,* 2013), although these are less common than generalization in other contexts. Despite the lack of studies looking into the generalization of latent inhibition, there is reason to suspect that latent inhibition should undergo generalization to the same extent as stimuli taught through association. Inhibitory responses in general are important in delineating the boundaries of cognitive discrimination tasks (Vervliet *et al.,* 2010), of which generalization is one such task. Additionally, inhibitory phenomena may form an association with the background context (Bouton *et al.,* 1999) or form a connection with the process of having directional resources diverted from it (Lubow, 2005), which are modified from prior models of inhibitory responses (Rescorla & Wagner, 1972) based on the sum of cue saliences (the rate of change for associative strength). Similar cognitive processes should affect latent inhibition as other associative phenomenon and generalization is known to occur for both risk (Griffin *et al.,* 2001; Smith *et al.,* 2008; Davis *et al.,* 2012; Chivers & Ferrari, 2013) and reward (see Bouton *et al.,* 1999 for review) associations. Learning about how latent inhibition is generalized could help to elicit properties about both cognitive processes in ecologically relevant contexts.

Generalization gradients can change depending on how the underlying information quality was altered, such as through steepening (Hearst & Poppen, 1965), flattening (Bouton *et al.,* 1999), or both steepening-like and flattening-like phenomena (Ferrari *et al.,* 2016). Since generalization gradients usually measure from an asserted baseline gradient (Hearst & Poppen, 1965; Bouton *et al.,* 1999), whether a gradient steepens or flattens could depend on which gradient is chosen as the representative one, though there might also be some mechanistic differences between the two alterations instead of one being the inverse of the other. Peak shifts are also known to alter generalization gradients, which is when the greatest response occurs for a novel stimulus rather than a trained stimulus due to the relation between to or more previously trained stimuli (Gamberale & Tullberg, 1996; Lynn *et al.,* 2005).

This study explored the effects of cognitive resonance during early development on the alteration of safety generalization gradients. This was done by asking the following four

questions: 1) Can embryonically exposed tadpoles form a generalization gradient from latently inhibited brook trout cues? 2) What does the generalization gradient look like? 3) Is the generalization gradient different from the gradient formed by larval exposed tadpoles? 4) In what way does the generalization gradient differ? There are multiple predictions on exactly how the gradient will change and there is no theoretical backing to prefer one to the other. Flattening is expected to occur in the embryonically trained tadpole generalization gradient if the sensitive period has little additional effect and time is the only factor. But flattening is expected to happen in the larval trained tadpole generalization gradient if the embryonically obtained information has enough reduced forgetting to slow the rate of flattening in the embryonically exposed tadpoles. Steepening is expected to occur in the embryonically trained tadpole generalization gradient if embryonic information is perceived as more informative, while the larval trained tadpole generalization gradients should steepen if more recent information is perceived as more relevant. Finally, it is possible that both processes interact, generating a concurrent broadening and sharpening phenomenon in one of the groups of tadpoles.

3.3: Methods:

3.3.1: Experimental Overview:

In order to test the effects of sensitive periods on the generalization gradient in tadpoles, a fully crossed 2 x 2 x 5 factorial experiment was designed with the following factors: ontogeny (2 levels: embryonic and larval), conditioning (2 levels: brook trout predation pre-exposure and control), and cue (5 levels: brook trout, splake, tiger trout, rainbow trout, and goldfish). The methods used in Experiment Two are modified from the methods used in Experiment One (Chapter Two). Tadpoles were collected at the same time as in Experiment One, and the same odours from the same fish were used. Following are a list of changes from the methods presented in Experiment One.

3.3.2: Treatment:

Four clutches of eggs were collected and each clutch was split into four equal-size sub-clutches the following day. Each sub-clutch was assigned to a 7 L pail (24 pails in total) containing 2 L of well water. Pails were arranged in 4 columns and 4 rows. Eggs were left in the pail for two days. On day two post-fertilization, pails assigned to the embryonic safety treatment were treated with 20 mL of brook trout odour six times within a 24-hour period. Eggs in the embryonic water treatment and both larval treatments were conditioned with an equal volume of water to maintain disturbance regime. Ten days post-hatch, tadpoles assigned to the larval safety treatment were treated with 20 mL of brook trout odour six times within a 24-hour period. The larval water treatment and both embryonic treatments pails were treated with 20 mL of well water to maintain disturbance regimes. Water changes were performed two hours after treatment.

Two days prior to the start of testing, 16 trays containing 30 cups were set up in a random arrangement in a nearby location. Two tadpoles from a selected tray were assigned to each cup, with one clutch per tray. Tadpoles were given 24 hours to acclimate to the cup environment before treatment applications. Tadpoles were then conditioned with one application of 20 mL brook trout odour, 20 mL splake odour, 20 mL tiger trout odour, 20 mL rainbow trout odour, or 20 mL goldfish odour immediately following these conditionings, each cup was treated with 10 mL of alarm cues. Five cups of each PO+AC pairing were performed per tray. An additional five were conditioned with brook trout and alarm cues. These tadpoles were to be tested with

well water to ensure that latent inhibition had occurred. Water changes occurred approximately two hours post-conditioning.

3.3.3: Testing:

Tadpoles were tested between 24-48 hours post-conditioning. Tadpoles were tested over the course of two days. Trays containing four rows of five cups were laid out in the open away from shadows that might cause a disturbance in the tadpoles. All cups in a tray received the same test odour. Conditioning treatments were assigned randomly to a row within a testing tray. Pails were chosen such that a testing tray would contain only tadpoles from the same clutch. Observers were blind to the conditioning treatment and the test cue. Each tadpole was given approximately 45 minutes to acclimate to the conditions of the cup. For four minutes pre-test, observers counted the number of line crosses each tadpole made across a line through the middle of the cup to serve as a baseline. A single line cross was defined as the entire body length of the tadpole crossing from one side to the other. Any tadpole that did not have a pre-test baseline line cross of 10 or greater was considered inactive and not recorded for the study. A subsample of tadpoles from the first day of testing that were considered inactive were removed and slated for retesting 48 hours after the original test day. Observers were then instructed to slowly inject 5 mL of the assigned test stimulus into each cup directly after the pre-test observation period. After a period of approximately one minute, a four-minute post-test line cross observation period was performed (960 tadpoles total). Tadpoles were also measured between 0-42 hours post-observation period in order to run body size as a covariate. The length of each tadpole was measured from head to tail.

3.3.4: Statistical Analysis:

3.3.4.1: Test for Successful Latent Inhibition: An activity metric was defined as $(Post - Pre)/Pre$ and used for all behavioural statistical analyses. A prior analysis using pre-injection activity and removing only tadpoles with pre-injection line crosses less than 10 (1 case) revealed a significant effect of conditioning ($F_{1,8.9} = 12.335, p = 0.007$) with the controls showing lower activity, indicating a need to use proportional change in line crosses. This analysis included data used in both the test for successful latent inhibition as well as the test for latent inhibition generalization and was used to justify using $(Post - Pre)/Pre$ for both statistical analyses. An ANCOVA was performed comparing the tadpoles with water as the test

cue to the tadpoles with brook trout as a test cue. This data set was analyzed using a 6-way nested ANCOVA using a Type I Sum of Squares. Conditioning treatment (2 levels), ontogeny treatment (2 levels), and tested cue (2 levels) were included as fixed factors. Clutch was included as a blocking factor. Pail and conditioning cup were included as nested factors. Total length was included as a covariate. The model included all fixed main effects first, then all fixed effect 2-way interactions, then the fixed effect 3-way interaction, then clutch, then pail nesting ontogeny*conditioning, then conditioning cup nesting ontogeny*conditioning, and then total length. Any data with a pre-injection line cross of less than 10 that were accidentally recorded (0 cases) were removed from the data set. Any data with a proportional change in activity greater than 1.0 were also removed from the analysis (3 cases). All outliers greater than 1.5*IQR + Q3 or 1.5*IQR – Q1 were removed from the analysis (12 cases). The remaining tadpoles with either brook trout or water as the tested cue were analyzed (262 cases). Tadpoles that could not be assigned length measurements (20 cases) were not included in the covariate analysis of the ANCOVA but were included in the fixed factor analysis of the ANCOVA. To account for the multiple uses of the brook trout treatment group in both the latent inhibition check and the generalization gradient check, all pairwise comparisons with brook trout were analyzed using a Bonferroni correction at $\alpha=0.0023$. All other comparisons in the latent inhibition ANCOVA and any ANCOVA assumption tests were analyzed at $\alpha=0.05$.

3.3.4.2: Test for Latent Inhibition Generalization: The data were analyzed as a 6-way nested ANOVA with length as a regression factor using a Type I Sum of Squares. Conditioning treatment (2 levels), ontogeny treatment (2 levels), and tested cue (5 levels) were included as fixed factors. Clutch was included as a blocking factor. Pail and conditioning cup were included as nested factors to account for the non-independence of the conditioning treatments and the reversal learning trials, respectively. A test for the assumptions of an ANCOVA revealed a significant interaction between length measurements and treatment groups ($F_{1,19} = 1.676$, $p = 0.036$). Due to the interaction with treatment groups and the limited size range (10-19 mm at a millimeter level resolution), tadpole length was kept in the ANOVA as a random factor rather than a covariate. Due to this interaction, the test for successful latent inhibition and the test for latent inhibition generalization were analyzed using different random factors. The model included all fixed main effects first, then all fixed effect 2-way interactions, then the fixed effect 3-way interaction, then clutch, then pail nesting ontogeny*conditioning, then conditioning cup

nesting ontogeny*conditioning, and then total length. Any data that were accidentally recorded with a pre-injection line cross of less than 10 (1 case) were removed from the data set. Any data with a proportional change of greater than 1.0 (12 cases) were also removed from the analysis in order to provide an upper bound that matched the lower bound of -1.0. All outliers greater than 1.5*IQR + Q3 or less than 1.5*IQR – Q1 (30 cases) were removed from the analysis. The tadpoles with water as the tested cue were removed from the generalization gradient analysis (132 cases). The remaining 606 cases were analyzed. Tadpoles that could not be assigned a length measurement (36 cases) were excluded in the regression analysis of the ANOVA but were included in the fixed factor analysis of the ANOVA. All significant interactions were subsequently analyzed by splitting the data and analyzing the appropriate nested ANOVA and Tukey's HSD post-hoc tests. All statistical analyses were performed in SPSS V.25, with a significance level set at α=0.05.

3.4: Results:

3.4.1: Successful Latent Inhibition: A test for the assumption of non-interactions between clutch and treatments revealed a significant interaction ($F_{21,223}$ = 2.245, p = 0.002), so clutch was kept in the model as a random factor. A test for the assumptions of the ANCOVA did not reveal a significant interaction between length and treatment groups ($F_{7,207}$ = 1.99, p = 0.06). An inspection of the residual plots did not reveal a significant deviation from the parametric assumptions of an ANCOVA. None of the random factors had any influence on the proportional change in activity. No three-way interaction between ontogeny*conditioning*cue was found ($F_{1,124.1}$ = 1.22, p = 0.27). A significant interaction between conditioning and cue was found ($F_{1,119.6}$ = 36.44, p < 0.001). Splitting the data by conditioning and running the subsequent 5-way ANCOVA revealed a significant effect of cue on the control treatment ($F_{1,49.6}$ = 33.09, p < 0.001) but not the brook trout safety treatment ($F_{1,69.8}$ = 4.33, p = 0.04) using a Bonferroni correction at α=0.0023 for the brook trout safety treatment but α=0.05 for all other comparisons (Fig. 3.1).

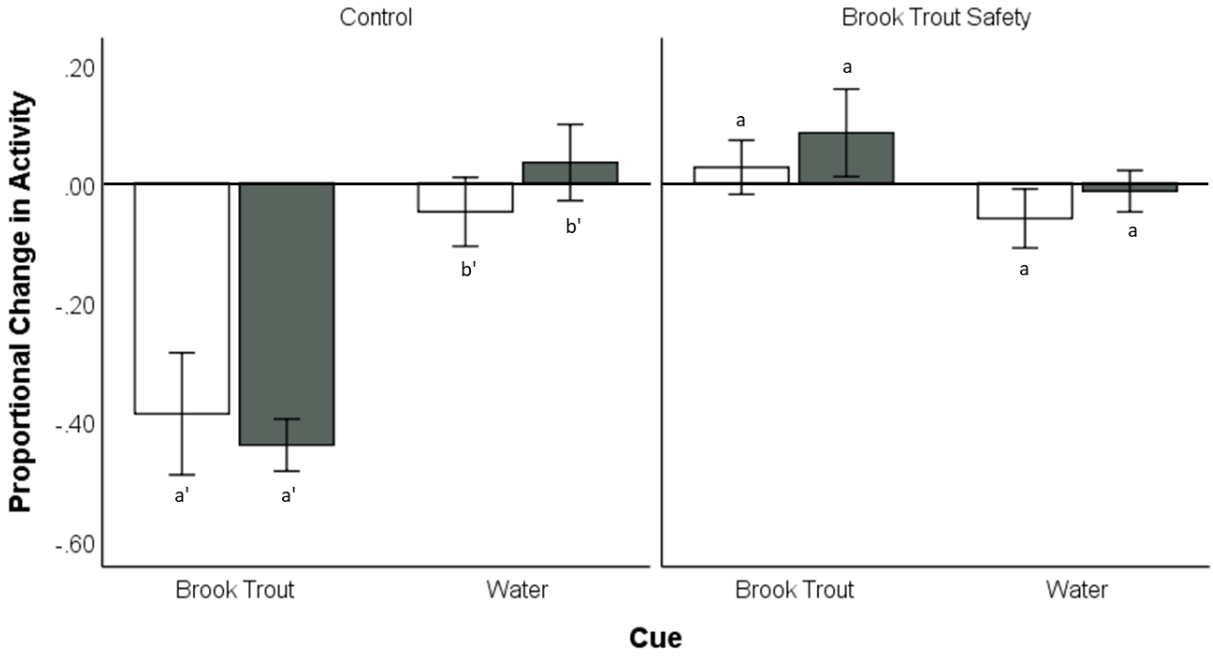

Fig. 3.1.

Mean proportional change in activity (± SE) for the test for latent inhibition, split by the control pre-exposure and brook trout safety pre-exposure. White bars indicate embryonically conditioned tadpoles that were either control tadpoles tested with brook trout (n=19), control tadpoles tested with water (n=30), brook trout safety tadpoles tested with brook trout (n=30), or brook trout safety tadpoles tested with water (n=37). Dark grey bars indicate larval conditioned tadpoles that were either control tadpoles tested with brook trout (n=28), control tadpoles tested with water (n=32), brook trout safety tadpoles tested with brook trout (n=35), or brook trout safety tadpoles tested with water (n=35). Letters indicate significant differences using an F-test with α=0.0023 for brook trout safety treatment and α=0.05 for the control treatment. Apostrophes indicate analyses that were performed separately.

3.4.2: Safety Generalization Gradient: A test for the assumption of non-interactions between clutch and treatments revealed a significant interaction between clutch and treatment group ($F_{57,532}$ = 1.648, p = 0.003), so clutch was kept in the model as a random factor. An inspection of the residual plots did not reveal a noticeable deviation from the parametric assumptions of an ANOVA. None of the random factors had any effect on the proportional change in line crosses. No significant three-way interaction between ontogeny*conditioning*cue was found ($F_{4,294.3}$ = 2.18, p = 0.07). A significant interaction between conditioning and cue ($F_{4,289.4}$ = 8.678, p < 0.001) and between ontogeny and cue ($F_{4,281.4}$ = 4.930, p = 0.001) was found. Splitting the data by conditioning and running a 5-way nested ANOVA revealed a significant interaction between ontogeny and cue for the brook trout safety group ($F_{4,149.7}$ = 5.574, p < 0.001) but not for the control group ($F_{4,145.5}$ = 0.98, p = 0.42). No further significant effects were found for the control group (Fig. 3.2). Splitting the data by conditioning and ontogeny and running a 3-way nested ANOVA with conditioning cup as the only nested factor revealed a significant effect of cue for both the embryonic safety treatment ($F_{4,73.5}$ = 6.997, p < 0.001) and the larval safety treatment ($F_{4,72.7}$ = 32.37, p < 0.001). A Tukey HSD post-hoc analysis revealed significant pairwise differences between the pairwise groups (Fig. 3.3).

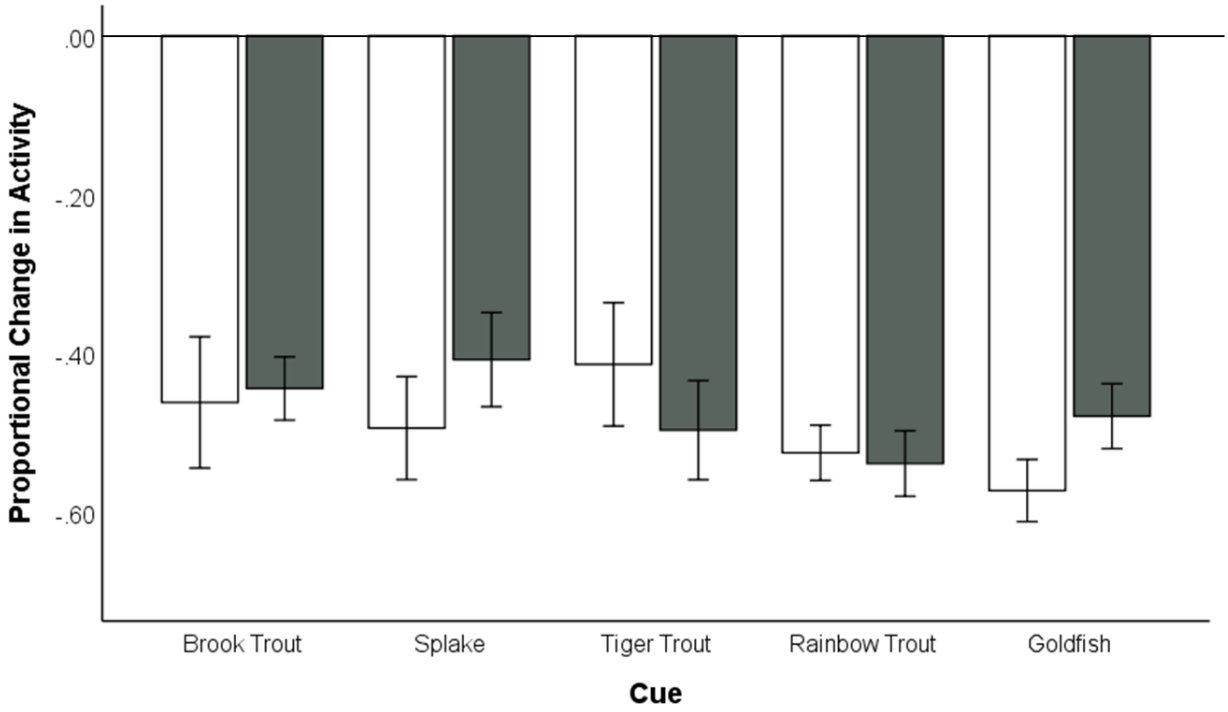

Fig. 3.2.

Mean proportional change in activity (± SE) for the control groups. White bars indicate embryonic control tested with brook trout (n=25), splake (n=23), tiger trout (n=25), rainbow trout (n=33), or goldfish (n=29). Dark grey bars indicate larval control tested with brook trout (n=31), splake (n=29), tiger trout (n=31), rainbow trout (n=35), or goldfish (n=32). No significant differences were found using Tukey's HSD or an F-test ($\alpha = 0.05$).

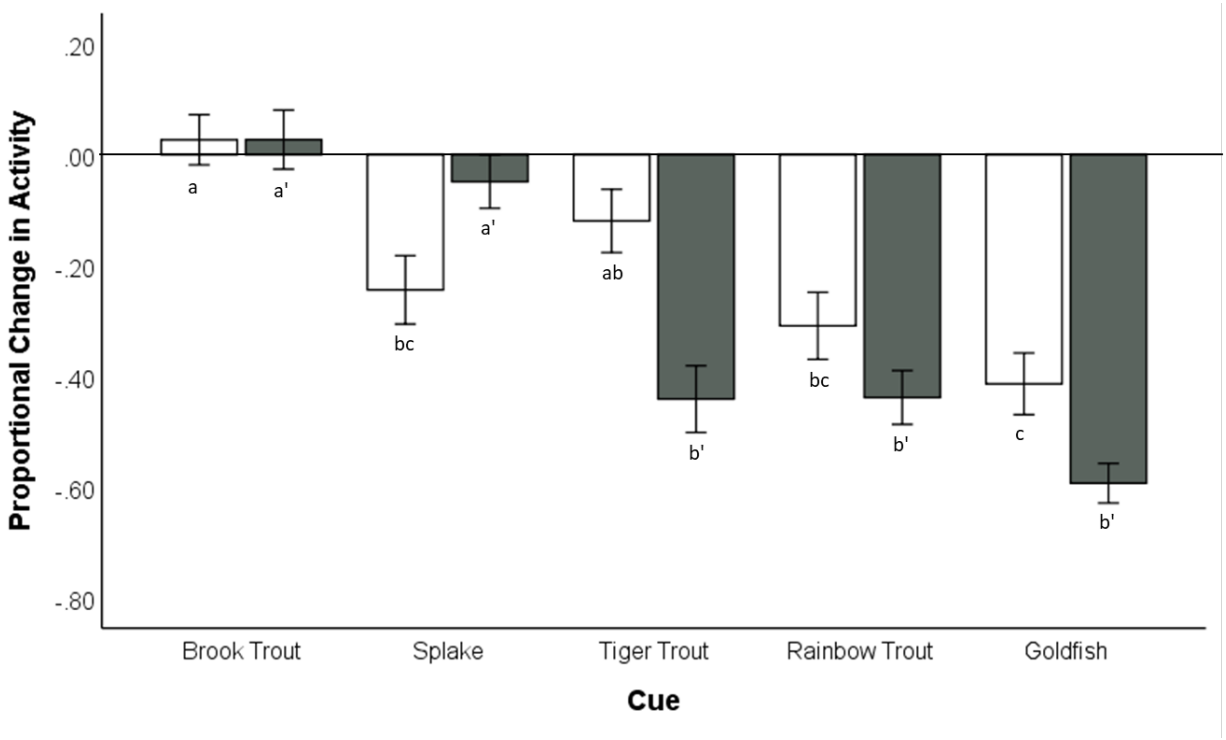

Fig. 3.3.

Mean proportional change in activity (± SE) for the brook trout safe groups. White bars indicate embryonic latent inhibition tested with brook trout (n=30), splake (n=28), tiger trout (n=32), rainbow trout (n=31), or goldfish (n=31). Dark grey bars indicate larval latent inhibition tested with brook trout (n=35), splake (n=31), tiger trout (n=32), rainbow trout (n=35), or goldfish (n=34). Letters indicate significant differences using Tukey's HSD ($\alpha = 0.05$). Apostrophes indicate analyses that were performed separately.

3.5: Discussion:

This study provides evidence that the timing of exposure to a latently inhibited cue results in an alteration of the generalization gradient. Embryonically exposed tadpoles (or ELI for embryonic latent inhibition) responded to tiger trout with statistically the same response level as the safety trained brook trout odour, whereas larval exposed tadpoles (or LLI for larval latent inhibition) did not (Fig. 3.3). LLI tadpoles responded to splake with the same response level as brook trout, whereas ELI tadpoles did not respond as strongly (Fig. 3.3). LLI tadpoles had two categories for assigning safety: those that did not undergo reversal learning and those that did (Fig. 3.3). LLI Tadpoles tested with brook trout and splake did not undergo reversal learning; but when tested with tiger trout, rainbow trout, and goldfish odour, the LLI tadpoles did undergo reversal learning. For ELI tadpoles, there was a continuum rather than two categories that formed. ELI tadpoles tested with brook trout did not undergo reversal learning, tadpoles tested with tiger trout underwent some reversal learning, tadpoles tested with splake or rainbow trout underwent more reversal learning, and tadpoles tested with goldfish underwent the most reversal learning. LLI tadpoles only recognized brook trout or intra-genus hybrids as safe, but recognized them as equally safe. Meanwhile, ELI tadpoles show the ability to assign different safety values to the various trout species. The ELI tadpoles appear to have assigned the inter-genus hybrid the same safety value as the taught brook trout, but not the intra-genus hybrid (Fig. 3.3). This cannot be explained by embryonically exposed tadpoles being less cautious around splake than larval exposed tadpoles as neither the embryonic water controls nor larval water controls (which underwent identical treatment procedures) did not show any difference between any of the treatment groups (Fig. 3.2).

This study is the first to conduct an in-depth look at the shape of the generalization gradient for safety related information in wood frog tadpoles. While risk related generalization information is common (Ferrari *et al.,* 2008; Ferrari *et al.,* 2009; Chivers & Ferrari, 2013; Ferrari *et al.,* 2016), safety related generalization information in aquatic systems is less known (Brown *et al.,* 2011; Ferrari & Chivers, 2011; Chivers *et al.,* 2013). Studies that have performed both risk and latent inhibition generalization in the same study have overall shown similar levels of generalization between risk association and latent inhibition (Ferrari & Chivers, 2011; Chivers *et al.,* 2013). In Brown *et al.* (2011), there was a difference in the level of generalization between risk association and latent inhibition for juvenile rainbow trout due to the use of a fourth species

within the gradient. Brown *et al.* (2011) showed that latent inhibition is generalized less than risk association using the same test gradient for both risk and safety generalization in the same study. Further comparisons between the gradients formed in Experiment One and Experiment Two in this thesis will be elaborated on in Chapter Four. However, the two experiments were performed on different days and so cannot be statistically compared. More studies should be performed to allow direct comparisons between risk generalization and latent inhibition generalization.

Comparisons can be made between the generalization gradient in this experiment and from established risk association generalization gradients, assuming the generalization process is domain general. In three studies (Chivers *et al.,* 2015; Ferrari *et al.,* 2016; Chivers *et al.,* 2016), risk associations were not transferable between brook trout and rainbow trout. In Ferrari *et al.* (2016) and Chivers *et al.* (2016), rainbow trout was the odour that was taught as risky and brook trout was a tested odour that was not generalized into. In Chivers *et al.* (2015), tiger trout was the odour trained as risky and both brook trout and rainbow trout were tested, but only brook trout was generalized into and rainbow rout was not generalized into. These studies indicate that rainbow trout and brook trout should be considered phylogenetically distant enough to not be generalized into, but my study suggests that this pattern is only true for larval exposed tadpoles and not for embryonically exposed tadpoles (Fig. 2.2, Fig. 3.3). The LLI generalization gradient are similar to the patterns observed with risk-associated generalization gradients (Fig. 3.3), which is somewhat expected given that all of the aforementioned studies used larval tadpoles instead of embryos for the initial pre-exposure. Interestingly, the ELI tadpoles were able to partially incorporate safety information from brook trout to rainbow trout, similar to the degree of generalization to the risk associated information in Experiment One. Behaving as if something is not safe when it is safe will result in lost energy; behaving as if something is not risky when it is risky will result in injury or death. As such, risk generalization is expected to be broader than safety generalization; but this was not the case for ELI tadpoles.

Unexpectedly, ELI tadpoles seem to have generalized to the more distantly related tiger trout than the more closely related splake (Fig. 3.3). While splake and tiger trout were not different from each other, splake and brook trout were different from each other while tiger trout and brook trout were not (Fig. 3.3). One possibility for this unexpected outcome is that flattening of the generalization gradient over time resulted in more distant stimuli increasing in

responsiveness and more closely related stimuli decreasing in responsiveness (Bouton *et al.,* 1999), with the increase in responsiveness to distant stimuli happening first and the decrease in responsiveness to similar stimuli happening at a later point in time. Alternately, it could represent a similar phenomenon to a peak shift, which is where the optimal response shifts to a novel stimulus and away from the originally trained stimulus (Purtle, 1973). Under this paradigm, tiger trout would appear to be shifting to become the new safety peak (Fig. 3.3). However, peak shifts normally occur during stimulus discrimination training and usually require exposure to three stimuli: two trained stimuli and the test stimulus (Gamberale & Tullberg, 1996; Lynn *et al.,* 2005). Another possibility is that the maternal and paternal factors are different between tiger trout and splake, resulting in different degrees of generalization (Chivers *et al.,* 2015). It could be that some factor about the tiger trout odour itself made it easier to learn as safe or that the splake odour was easier to learn as risky, although why the larval tadpoles did not also show this response pattern is not addressed by this explanation (Fig. 3.3).

LLI tadpoles respond to the intra-genus hybrid (Fig. 3.3) and respond with caution to more distant odours, which mitigates Type I error. This could be adaptive as the organisms within the pool and between pools may vary (see Colburn *et al.,* 2008 for review), so forgetting all but the most specific information regarding safety frees up cognitive resources to be used elsewhere. This allows for greater stimulus discrimination as it serves as an anchor for peak shift (Gamberale & Tullberg, 1996; Lynn *et al.,* 2005) against which other stimuli can be tested. Additionally, increased information along the stimulus axis can result in narrowed generalization gradients (Ghirlanda & Enquist, 2003; Ferrari *et al.,* 2016), so latent inhibition could help to shape risk associated generalization gradients as well by providing a complementary generalization gradient. However, ELI tadpoles might be in the process of forgetting as they had more time to forget the information, hence the increased responsiveness to more distant stimuli. Such adaptive forgetting (Kraemer & Golding 1997; Ferrari *et al.,* 2010a; Gonzalo *et al.,* 2013) would provide ELI tadpoles with a greater advantage if the organisms in the pool vary (see Colburn *et al.,* 2008 for review). Maintaining safety related information might be detrimental if the individual is only safe as an egg or smaller tadpole and become increasingly dangerous as the tadpole gets larger (Anderson & Brown, 2009). However, the observed generalization gradient (Fig. 3.3) is unlikely to be a result of time alone, as there was still a heightened response to the

initially trained cue, which is not predicted by a purely temporal flattening of the generalization gradient (Bouton *et al.,* 1999; Wiltgen & Silva, 2007).

This study shows that embryonic safety generalization gradients are different from larval safety generalization gradients. Safety information taught outside a sensitive period created two distinct categories: intra-genus hybrids were safe, everything else was novel (Fig. 3.3). Safety information taught within a sensitive period formed a more gradual gradient with no clear categories separating either of the hybrids or the remaining trout (Fig. 3.3). These differences in the generalization gradients are important not only for understanding how generalization gradients form (Ferrari *et al.,* 2016), but how safety related information is generalized (Hazlett, 2003). Generalizing safety allows organisms to know when stress levels can be lowered and other biologically relevant activities can resume (Lima & Bednekoff, 1999). If threat levels are always perceived as being high, the energetic demands of growth might not be met and result in further susceptibility to predation threats, especially to predators with upper size limits for prey capture (Semlitsch, 1990). Finally, as tadpoles would have likely evolved under environmental conditions with a more complex olfactory environment than the experimental apparatus would have provided (see Colburn *et al.,* 2008 for review on ephemeral pools), it is possible that early exposure to latently inhibited olfactory cues provide an appropriate baseline for other generalization gradients to form.

Chapter 4: General Conclusions:

4.1: Summary of Findings:

Both experiments presented herein indicate that generalization gradients are affected by cognitive resonance and are altered based on the ontogenetic timing of the first exposure. In Experiment One, risk related information first presented during embryonic development resulted in a broader generalization gradient than the same information first presented post-hatching. When brook trout was taught as risky, risk association was generalized to all members of the Salmonidae family that were tested in the embryonic treatment but only to the brook trout hybrid species in the larval treatment. The embryonically taught tadpoles were more likely to respond to any stimulus as risky even in the control treatments when compared to the larval taught tadpoles, possibly contributing to the increased responsiveness to the more distantly related rainbow trout. In Experiment Two, safety related information first presented during embryonic development resulted in a broader generalization gradient than information first presented as larvae. When brook trout was taught as safe, safety was generalized to the all the members of the Salmonidae family that were tested in the embryonic treatment, but only to the intra-genus brook trout hybrid group in the larval treatment. The safety control groups showed no differences from each other.

For both experiments, the generalization gradient for the embryonic trained tadpoles appears to be less categorical than the generalization gradient of the larval trained tadpoles. In Experiment One, this was demonstrated by the overlapping post-hoc subsets for splake and tiger trout in the embryonically trained generalization gradient compared to the mutually exclusive post-hoc subsets between tiger trout and rainbow trout in the larval trained generalization gradient. In Experiment Two, this was demonstrated by the overlapping post-hoc subsets for splake, tiger trout, and rainbow trout in the embryonically trained gradients compared to the mutually exclusive post-hoc subsets between splake and tiger trout in the larval trained gradients. In both experiments, the larval treatment had two clear delineations. For risk, the two categorizations were the brook trout hybrids and the ungeneralised stimuli. For safety, the two categorizations were the brook trout intra-genus hybrid group and the ungeneralised stimuli. Unexpectedly, the more distantly related tiger trout was generalized as safer than the more closely related splake in the embryonic group, with splake and rainbow trout falling into the

same moderately risky subset. Further studies will need to be conducted to discern the primary driving factors.

4.2: Understanding the Effects of Cognitive Resonance and Future Research Directions:

4.2.1: Process Oriented Explanations for Gradient Alterations:

By comparing both experiments, the underlying mechanism of cognitive resonance and its effects on generalization can be speculated on, as well as the causes and effects of cognitive resonance across other cognitive processes. Potential explanations for how the generalization gradient was altered as a result of the cognitive resonance can fall into three non-mutually exclusive subsets: flattening, steepening, and peak shift.

4.2.1.1: Flattening: In these studies, flattening would be expected to occur for the embryonically trained associations, as the embryonic tadpoles had 10 extra days to forget the information presented to them, and the embryonically trained tadpoles in both the risk associated cue and the safety cue had the same extinction process as the larval trained tadpoles. Flattening would also be expected in the larval trained associations due to the gap between the last training session and testing, but not as much as in the embryonically trained group. Depending on the paradigm being used, flattening due to time could be either due to adaptive forgetting of irrelevant information (Bouton *et al.,* 1999; White, 2001) or increased sensitivity to incorrect stimuli (Bouton *et al.,* 1999). This explanation would be the most plausible if the sensitive period had no real effect on generalization development and that the alteration was a result of time alone. The data collected do not appear to support temporal flattening of the generalization gradient as a complete explanation. In both experiments, the embryonic group seems to have held on to a generalization gradient for a longer duration than that of the larval group with no comparative decrease in responsiveness to similar stimuli, while the larval group showed some alteration away from previously described generalization gradients (Ferrari & Chivers, 2009a; Ferrari *et al.,* 2009; Chivers *et al.,* 2015; Ferrari *et al.,* 2016). However, none of the prior studies used exactly the same phylogenetic gradient as the current one, nor the same ontogenetic stages nor temporal gaps. This makes direct comparisons somewhat difficult, so more standardization in the phylogenetic gradients should be used in future studies.

Maintenance of the gradient would follow suit from the original paper describing cognitive resonance, since it showed that information obtained embryonically is maintained for

longer (Ferrari *et al.,* 2019). The generalization gradient could also be maintained due to reliance on the shared cognitive resource of memory (Sih & Giudice, 2016), memory attentional resources (Kiyonaga & Egner, 2014), or on the shared cognitive process of forgetting (White, 2001; Vlach & Kalish, 2014). The embryonically trained generalization gradient could take longer to flatten, unless the sensitive period also affected the process of generalization in a counterbalanced way. The amount of time required to see substantial flattening using this paradigm and study system are not well known. Future studies should look at how the embryonic and larval generalization gradients degrade as a function of time directly in order to account for these temporally bound alterations.

4.2.1.2: Steepening: Either treatment could have experienced steepening in these two studies depending on the strength of cognitive resonance on generalization and whether flattening was occurring. If the effect of cognitive resonances was weak and flattening was minimized, there would be an expected steepening for the larval group since the information would be more recent and therefore considered more relevant. More recent information will usually be considered more relevant unless substantial time has passed and forgetting increases (Kraemer & Golding, 1997; White, 2001). If the sensitive period had a sufficiently strong effect or the gelatinous sheath containing the egg mass allowed for a longer effective exposure time, then the embryonic tadpoles would be expected to show steepening as a result of the perceived increase in information quality. Training during the sensitive period also takes up a greater proportion of the individual's experienced life than the same amount of time outside of the sensitive period. This could have a similar effect as a perceived increased number of training sessions for the embryonically trained tadpoles, resulting in the same kind of generalization gradient sharpening found in Ferrari *et al.* (2016).

These studies seem to indicate that steepening did not occur in the embryonic groups but may have contributed to some alterations in the larval groups. In both experiments, the embryonic groups had the broadest generalization gradients, while the larval groups developed two distinct categories: one group that was generalized into and one that was functionally novel. This categorization in the larval groups is similar to the sharpening described by Ferrari *et al.* (2016), where repeated exposures to a trained odour resulted in more clear-cut categorization than did a single session. While steepening is already associated with increased information

quality, steepening is an unlikely explanation as steepening uses an increase in stimulus concentration as the indicator of increased quality (Ferrari *et al.,* 2009), which did not occur during these experiments. Comparisons of generalization gradients brought on by alterations of alarm cue concentrations rather than number of exposures has not been explored for wood frog tadpoles and would need to be explored more thoroughly in order to rule out steepening as explanation or to provide a mechanistic distinction between steepening and the sharpening phenomenon noted by Ferrari *et al.* (2016).

4.1.2.3: Peak Shift: While not an expected outcome of the experimental setups used, peak shifts can also alter generalization gradients. Peak shift is considered a discrimination phenomenon because it almost always requires three stimuli to test: two to train (either both excitatory or one excitatory and one inhibitory) and a third to serve as a probe (Lynn *et al.,* 2005). As designed, neither experimental setup could show a peak shift, though the latent inhibition test does incorporate two of the three required components (both an excitatory and inhibitory stimulus, but no third probe) and showed an unexpected shift in generalizing the latent inhibition towards tiger trout. Such a peak shift analogue has been recorded once in wood frog tadpoles when testing cues from hybrid trout species using the maternal and paternal lines (Chivers *et al.,* 2015), where the maternal line showed increased responsiveness. It could be possible that the maternal line for tiger trout odour had a stronger influence than in splake. This explanation is less likely since splake all have the same maternal/paternal pairing of male brook trout x female lake trout (Chevassus, 1979), and tiger trout with female brook trout x male brown trout parental lineages suffer higher mortalities than the reverse parental cross (McKay *et al.,* 1992). There is therefore little reason to suspect one hybrid had a different maternal/paternal affect. Also, it does not fully address why the alteration in Experiment Two only happened to the embryonic group and not the larval group despite being exposed to the same odour stock.

A more likely explanation is the lack of knowledge on how wood frog tadpoles interpret the hybrid trout species. The only study found which has looked into wood frog tadpole reactions to tiger trout is Chivers *et al.* (2015), few have looked into wood frog tadpoles to brown trout (see Ferrari *et al.,* 2016), and no study found looked into reactions of wood frog tadpoles to splake or lake trout. These studies have shown that neither tiger trout nor splake are recognized as inherently as risky, which is the first instance of tiger trout being tested for

potential innate associations. It is unknown if splake is easier to learn and/or retain an association with after initial learning event. Alternatively, tiger trout may be harder to unlearn an association with after the initial learning event. This explanation is unlikely, since Experiment Two showed that larval tadpoles did not have the same peak shift in the latent inhibition generalization gradient. More studies would need to be done with wood frog tadpoles to tiger trout and splake odours to rule this out completely.

4.2.2: Comparison of Generalization Gradient Differences:

In the risk association experiment, the larval group generalized to both splake and tiger trout. The comparable larval group in the safety experiment only generalized to splake, not tiger trout. This is an expected outcome as risk and safety have two different relationships to false positive and false negative assessments of stimuli (Wiley, 1994 through Lynn *et al.,* 2005; Lima & Bednekoff, 1999). Energetically costly antipredator responses will be undertaken unnecessarily if there is a false positive in risk association, but the individual could be injured or killed if there is a false negative in risk association. Conversely, a false positive for safety association could also lead to injury or death, but a false negative only leads to an energetically costly and unnecessary antipredator response. Safety related information should therefore be more specific and generalized into less than risk related information. The embryonically trained tadpoles appear to go against this trend, with both experiments showing generalization to rainbow trout. It could be that the cognitive resonance affected the process of generalization directly, making it more domain general and less domain specific. Cognitive domains are the sets of types of information and responses that a cognitive function can apply to (Fodor, 1983 as stated by Spunt & Adolphs, 2017), with cognitive functions being steps in information processing (Cauchoix & Chaine, 2016). If cognitive resonance affected the degree of domain specificity for the generalization process, then the embryonically trained tadpoles could have a more generalization process that is less domain specific, while the larval trained tadpoles could have a more domain specific generalization process. Performing experiments using different modalities, checking for domain specificity in excitatory or inhibitory contexts, and checking for an actualized asymmetry between interpretations for safety and risk related information will also need to be done in order to disentangle the effect of cognitive resonance on generalization.

4.2.3: Non-Cognitive Process Oriented Explanations for Generalization Gradient Alterations:

There are other explanations for the observed differences that take into consideration proximate mechanisms instead of strict gradient alterations. If cognitive resonance is affecting memory directly (either through encoding, storage, recall, or attentional alterations), then associative learning and generalization later in life would be affected indirectly through the alteration to a shared cognitive resource (Kiyonaga & Egner, 2014; Cauchoix & Chaine, 2016). Conversely, the memories might not change but the forgetting function may be altered. In Ferrari *et al.* (2010a) it was suggested that generalization gradients formed from low cue concentrations are forgotten faster in order to free up cognitive resources from irrelevant information, allowing more recent information to be prioritized and weighted accordingly due to perceived accuracy (Kraemer & Golding, 1997). This adaptive forgetting (Kraemer & Golding, 1997; White, 2001) can even result in complete and irreversible forgetting of fear information in humans and rodents (Hartley & Lee, 2015). If cognitive resonance was altering the forgetting function, associative learning and generalization would be affected by a shared process. Finally, forgetting can be viewed as a type of generalization (Vlach & Kalish, 2014), so any direct effect on any aspect of generalization that is domain general would also effect forgetting. These possibilities could be studied by examining other contexts, such as training under a risky or safe association then testing reward circuits to see if memory or forgetting are affected across the board.

Embryonic tadpoles are contained within a gelatinous layer, which is already known to be impermeable to dyes with a large mean molecular mass, have diffusion rates that depend on the arrangement of the eggs, and have species differences (Pinder & Friet, 1994). If the trained odour is a complex chemical mixture, perhaps only some of the odorants are reaching the embryo through the gelatinous layer, so the two generalization gradients are generated from two different pieces of information. Additionally, odorants that manage to diffuse into the gelatinous layer may not be able to diffuse out as easily, resulting in increased effective exposure times. The chemical composition of brook trout odours and wood frog alarm cues are not completely known. The permeability of the gelatinous layer is not entirely known, especially with regards to the olfactory stimuli used in these experiments. As such, the components of either brook trout

odour or alarm cues that managed to reach the embryonic tadpoles cannot be determined and any potential effects remain unknown.

The degree that embryonic tadpoles use olfactory information is also a matter of debate. Frog eggs are known to hatch faster as a result of predation pressure (see Warkentin, 2011 for review), but the role this plays in wood frogs is not as clear as with other frog species. It is also unclear to what extent egg derived cues and larval derived cues are chemically different, which itself may be species specific (Touchon *et al.,* 2006; Warkentin, 2011). If egg derived cues are more ecologically relevant for embryos than larval cues, the use of larval alarm cues may have altered the degree of ecological relevance in these experiments. Additionally, embryonic neurological development usually starts at the same time as the first embryonic exposures in embryonic learning experiments in tadpoles (Gosner, 1960; Supekar & Gramapurohit, 2017; Ferrari *et al.,* 2019). A less developed neurological system might be less capable of recognizing and encoding complex chemical mixtures when compared to more developed neurological system, resulting in a decrease in the complexity or completeness of information received.

Finally, it could also be that the embryonic treatment groups are more similar to proper cognitive development in tadpoles than the controls. In both experiments, the experimental groups were exposed to one additional odour than the control groups: alarm cue for Experiment One and brook trout for Experiment Two. Background odours were kept to a minimum due to the physical separation of the tadpole from their natal pond, the use of a more controlled water source, and netting to prevent predation efforts. Since tadpoles would have likely evolved under conditions that would likely have far more odours and other indicators present than those of the controls for the experiments in this thesis (see Colburn *et al.,* 2008 for review on ephemeral pools), it could be that the controls did not develop a proper olfactory system. Due to the alter exposure to additional cues, it is also possible that the larval trained tadpoles did not fully develop a proper olfactory system as well. As such, it might be more prudent to ask what mechanisms lead to the larval treatment group to have an altered gradient.

4.3: Broader Impacts:

4.3.1: Expanding Model Systems:

This thesis aids in understanding wood frogs as model systems for cognitive ecology. The increased generalization gradient in embryonically trained tadpoles provides some method

of accounting for any potential inter-individual variability in generalization for wild caught tadpole populations. As mentioned above, the research presented in this thesis raises many questions on how generalization gradients form and degrade in wood frog tadpoles. To ensure that these generalization gradient alterations can provide a foundation for future cognitive ecology theory, any affects on ultimate fitness would need to be tested (Cauchoix & Chaine, 2016). This thesis also demonstrates the usefulness of the brook trout hybrids species complex as an ecologically relevant gradient for the olfactory generalization of aquatic prey species and showed for the first time that tiger trout is not innately recognized by wood frog tadpoles as either risky or safe. Future studies should also aim to incorporate more of the phylogeny of the group into the gradient itself. The taxonomic relations between all species used in this thesis are well known (Murata *et al.,* 1993; Crespi & Fulton, 2004, Horreo, 2017), and there are well known methods of incorporating phylogenetics into data analyses such as Blomberg's K and the lambda metric (Blomberg *et al.,* 2003; MacLean *et al.,* 2012; Münkemüller *et al.,* 2012). These methods are primarily for transforming collected data, but could still prove useful for modifying the tested axis as well. Proper weighting of the distances between comparable points for the olfactory cues could lead to the detection of potentially significant differences that would otherwise be masked or lead to greater predictive power for how a novel stimulus might be generalized. Testing new species within the complex such as Lake Trout or Brown Trout could also help to create an even more thorough gradient.

4.3.2: Understanding Sensitive Periods Outside of Early Ontogeny:

While certainly the most studied sensitive period (Kinsella & Monk, 2009; Fox *et al.,* 2010; Leppänen & Nelson, 2012; Fawcett & Frankenhuis, 2015; Panchanathan & Frankenhuis, 2016), early ontogeny is not the only sensitive period known. The onset of sexual maturity is another such sensitive period (Fawcett & Frankenhuis, 2015; Fuhrmann *et al.,* 2015) and adolescence serves as a sensitive period for specific types of social information in humans (Fuhrmann *et al.,* 2015). While it is not known if wood frog tadpoles would have a truly comparable second sensitive period, the onset of metamorphosis might provide a surprising analogue and would be interesting to see what information passes through such an ecological niche shift (Trokovic *et al.,* 2011). The post-overwintering thaw that wood frogs can undergo, due to their ability to handle extracellular freezing, could also form a third sensitive period that would be interesting to check (Storey & Storey, 1984). These two potential sensitive periods

could also be used to determine if cognitive resonance has the same affects on the same cognitive processes in different sensitive periods or if different sensitive periods result in fundamentally different resonance-type phenomena, which could then be transferred to other sensitive periods of interest in other systems of interest once the fundamentals of cognitive resonance have been described.

4.3.3: Relations to Adaptive Heuristics and Other Cognitive Systems:

Cognitive biases are rulesets that result in an individual making mathematically non-optimal decisions that do not reflect true self interest or the true state of the world (Tversky & Kahneman, 1974; Mathis & Steffen, 2015). Cognitive resonance appeared to generate several cognitive bias-like states in the embryonic tadpoles (Ferrari *et al.,* 2019). This includes the anchoring fallacy or focalism, which is giving undo focus to the first piece of information presented, and conservatism or the regressive bias, which is the failure to update event probabilities when new information is obtained (Tversky & Kahneman, 1974). These are examples of the availability heuristic, which is information with greater mental availability is given disproportionately greater weight (Tversky & Kahneman, 1974). The overgeneralization of both responses in embryonically trained tadpoles in these studies resembles the law-of-small numbers bias (Tversky & Kahneman, 1974), which is the discounting of the high variability of small samples. Since embryonically trained tadpoles have sampled the environment less than larval tadpoles, rare events may have been disproportionately included in the sample. Since the adaptive toolbox depends on environmental restrictions in information processing (Gigerenzer & Selten, 2002), cognitive resonance provides a long-term environmental restriction that promotes certain biases to be expressed under certain contexts. It is unknown at this time if these biases fix under other contexts or if there is an adaptive reason for why embryonic learning might promote certain biases over others.

This research can be applied to other biological systems, including chickens (Sneddon *et al.,* 1998), cichlids (Nelson *et al.,* 2013), and mites (Quesada & Schausberger, 2012) for early development research, and sheep/goats (Lubow & Moore, 1959), damselfish (Mitchell *et al.,* 2011), crayfish (Hazlett, 2003), minnows (Ferrari & Chivers, 2006), and rats (Tsakanikos & Reed, 2019) for latent inhibition. Of particular note are human systems which have shown a link between generalization and abstraction (Vlach & Kalish, 2014), latent inhibition (Lubow, 2005),

and early developmental effects (Hepper, 1996). If early ontogenetic experiences effect how an individual human generalizes information, as this study indicates, then such experiences may also affect the abstraction process in humans. Latent inhibition in humans has already been heavily linked to anxiety in rats (Tsakanikos & Reed, 2019) and is a known paradigm for linking several other symptoms in schizophrenia (Lubow, 2005). This thesis shows that exposure to latently inhibited cues during sensitive periods will result in more broadly generalized latent inhibition and potentially reduced anxiety or buffer from schizophrenic-type symptoms. It is worth noting that embryonic development is not usually seen as the primary sensitive period of interest in humans, but the *in-utero* environment can still alter fetal phenotype (Kinsella & Monk, 2009) and similar processes might be found in other sensitive periods.

4.3.4: Behavioural Conservation:

The field of behavioural conservation is the study of how behaviour can be used to aid in conservation efforts (Sutherland, 1998; Berger-Tal *et al.,* 2011). In recent years, cognition has been suggested as another aspect to include in conservation, as it can be useful for promoting successful reintroduction efforts, allow for successful habituation of human disturbance, prevent unwanted human-animal interactions, and is generally connected to behaviour (Greggor *et al.,* 2014). Pheasants are known to have different spatial reasoning abilities if reared with and without a spatially complex environment (Whiteside *et al.,* 2016), salmon show different antipredator responses depending on whether the population is captive reared for one or more generations (Jackson & Brown, 2011), and spatial learning in zebrafish depends on whether a complex spatial environment was present during early ontogeny (Roy & Bhat, 2016). Not every system analyzed showed cognitive differences between populations (Benhaim *et al.,* 2013). In sea bass, only behavioral metrics of velocity and swimming distance were affected, and reward learning circuits were not (Benhaim *et al.,* 2013). It is therefore important for captive populations to be exposed to all cognitive problems that would be encountered in a natural environment in order to ensure the maintenance of adaptive naturalistic responses, especially for reintroduction efforts (see Sutherland, 1998 for early review). Rainbow trout do show greater survival with predator odour pre-exposure in both in the captive settings and in the field (Mirza & Chivers, 2000), even though these fish had already hatched by the time of the first training session. This research indicates that exposing said rainbow trout as eggs may have allowed them to generalize the information more or hold on to the gradient for longer, increasing early

recognition of other potential predators and increasing survival rates more than later training sessions. Latent inhibition could also function the same way, but for recognizing safety instead of predation.

It is important to consider where such early pre-exposure may cause unintended consequences. It is known that antipredator responses in tadpoles work for some potentially invasive predators but not others (Ferrari *et al*., 2015). According to this thesis, embryonic training might result in too broad of a generalization gradient, such that pre-training may be worse than no training at all if the resultant response would otherwise decrease fitness. Safety would have the related problem, potentially resulting in an inability to learn an invasive predator that happens to be related to a known safe organism. Additionally, preferences can be influenced by parental factors and parental information is expected to be more heavily weighted if the environment is predictable enough (see Taborsky, 2017 for review with cichlids). Parental lines choose environments that are most similar to where they thrived or that is more similar to the environment they encountered as offspring, creating a situation where the offspring will develop a cognitive or behavioural phenotype that matches the parent. Maternal factors can also influence fitness, as has been recently demonstrated in juvenile male cichlids with maternal temperature acclimation (Fuxjäger *et al.,* 2019). Maternal lines that prefer captive environmental indicators may impart that preference to their offspring, overriding any benefit from cognitive resonance.

4.3.5: Other Plastic Responses:

Resonance-type phenomena are not the only phenomena that can restrict plasticity both within and between generations. Frameworks for cognition that rely on evolutionary theory over computational theory are still a relatively recent phenomenon, but have been developed by Mendelson *et al.* (2016) and are reliant on suites of characteristics being the units of selection (a similar idea follows from Stuart *et al.,* 2002 involving behaviour alone). Behavioural syndromes, cognitive syndromes, and cognitive styles (Sih & Giudice, 2016) are all methods of restricting the formation of suites of behavioural or cognitive characteristics. According to Sih & Giudice (2016), behavioural syndromes are suites of correlated behaviour, cognitive styles are individually favored heuristics complements, and cognitive syndromes are suites of correlated cognitive abilities and behaviours. Each of these would restrict plasticity in behavioral or

cognitive responses by applying a correlative structure. The predator recognition continuum hypothesis also addresses trade-offs with plasticity (Ferrari *et al.,* 2008; Ferrari & Chivers, 2009a), as plastic responses are expected when predator complements are variable and risk is low, while innate responses are expected when predator complements are stable and risk is high. The generalization gradient showed a plastic response to differential information presentation by having a different gradient shape for the embryonically trained tadpoles compared to the larval trained tadpoles, while the process of generalization induced a labile response through prompting (Experiment One) or preventing (Experiment Two) a risk avoidance behaviour to novel stimuli. Even though the Bayesian approximations of behaviour indicate an early opening of plasticity during sensitive periods (Fawcett & Frankenhuis, 2015), the maintained information that was originally shown in cognitive resonance (Ferrari *et al.,* 2019) and the generalization gradient changes in these studies indicate a loss of plasticity later in development.

4.4: Conclusions:

This thesis has demonstrated that the timing of the first exposure to both risk related and safety related information will alter the shape of the generalization gradient in wood frog tadpoles, following from the conceptual groundwork of cognitive resonance (Ferrari *et al.,* 2019). Wood frog tadpoles appear to have broader generalization gradients when taught information embryonically than when taught the same information larvally. In larval taught tadpoles, the generalization gradient was smaller for safety related information than risk related information, but this was not true for embryonically taught tadpoles. Both embryonic groups generalized information to rainbow trout whereas the larval groups did not, indicating a potential cognitive bias brought on by the existence of a sensitive period. Finally, tadpoles taught as larvae seem to have created a more categorical delineation, whereas embryonically trained tadpoles appeared to have more gradual gradients. This thesis has wide ranging implications, including providing some of the earliest evidence for cognitive resonance, providing an ecologically relevant cognition test for embryonic learning in tadpoles, and furthering the foundations of wood frog tadpoles as model systems in cognitive ecology.

Ethics Statement

All procedures performed herein were performed in accordance with the University Committee on Animal Care and Supply Animal Care Protocol 20060014 that was approved at the University of Saskatchewan.

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